THE
BIOLOGICAL BULLETIN

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THE MARINE BIOLOGICAL LABORATORY

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CONSERVATION OF SCHOLARLY JOURNALS

The American Library Association created this last year the Committee on Aid to Libraries in War Areas, headed by John R. Russell, the Librarian of the University of Rochester. The Committee is faced with numerous serious problems and hopes that American scholars and scientists will be of considerable aid in the solution of one of these problems.

One of the most difficult tasks in library reconstruction after the first World War was that of completing foreign institutional sets of American scholarly, scientific, and technical periodicals. The attempt to avoid a duplication of that situation is now the concern of the Committee.

Many sets of journals will be broken by the financial inability of the institutions to renew subscriptions. As far as possible they will be completed from a stock of periodicals being purchased by the Committee. Many more will have been broken through mail difficulties and loss of shipments, while still other sets will have disappeared in the destruction of libraries. The size of the eventual demand is impossible to estimate, but requests received by the Committee already give evidence that it will be enormous.

With an imminent paper shortage attempts are being made to collect old periodicals for pulp. Fearing this possible reduction in the already limited supply of scholarly and scientific journals, the Committee hopes to enlist the cooperation of subscribers to this journal in preventing the sacrifice of this type of material to the pulp demand. It is scarcely necessary to mention the appreciation of foreign institutions and scholars for this activity.

Questions concerning the project or concerning the value of particular periodicals to the project should be directed to Wayne M. Hartwell, Executive Assistant to the Committee on Aid to Libraries in War Areas, Rush Rhees Library, University of Rochester, Rochester, New York.

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PHYSIOLOGICAL OBSERVATIONS UPON A LARVAL EUSTRONGYLIDES

II. The Aerobic Respiration [1]

THEODOR VON BRAND [2]

In the first paper of this series (von Brand, 1938) the chemical composition of a larval *Eustrongylides* was studied to some extent. It was characterized by a high glycogen and haemoglobin content, whereas only a little fat was found. The different rates of glycogen consumption under aerobic and anaerobic conditions seemed to indicate that the worm, in contrast to *Ascaris* and several other parasitic worms, was adapted to an oxidative rather than an anoxidative type of metabolism. In order to carry the analysis of its metabolic processes one step farther, a study of its respiratory exchange was undertaken.

Material and Methods

All the larvae (probably the larva of *Eustrongylides ignotus* according to a personal communication by Dr. B. G. Chitwood) were extracted from cysts along the mesenteries of *Fundulus heteroclitus* and only medium-sized to large worms (40 to about 120 mgm.) were used. They were washed with saline, dried quickly on filter paper, weighed with an accuracy of 1 mgm. and placed in the respiratory vessels. From one to four worms (100 to 300 mgm. fresh tissue) were used in making a single determination. All the data given below for the gaseous exchange are based on the weight of the living worms immediately after isolation. The salt solutions used varied in the different series, and are mentioned below.

The experiments were carried out with manometers of the Warburg type, using vessels of about 18 cc. capacity, and conducted at a temperature of 37° C. This high temperature was chosen rather than the lower

[1] A contribution from the Department of Biology, The Catholic University of America, Washington, D. C.

[2] The author is indebted to the Elizabeth Thompson Science Fund for a grant towards the purchase of the respiration apparatus used in this investigation.

temperature to which the worms were accustomed in the fish, since the definitive host is quite certainly a warm-blooded animal and the parasite would normally live at the temperature prevailing in the glands of the fore-stomach of aquatic birds after leaving the cyst. To determine the respiratory quotients of the experiments summarized in Tables III and IV, the worms were kept first for two hours in the vessels without KOH, then for the same length of time in the same vessels after addition of KOH. This procedure was permissible, since preliminary experiments had shown that the manometer readings, both for CO_2 and O_2, remained sufficiently constant during such periods. The respiratory quotients of the experiments summarized in Table V, on the other hand, were determined according to Warburg's indirect method, since it was necessary to determine here the RQ's in shorter intervals throughout the course of the experiments. In all cases the readings were taken in half-hour intervals, and the first reading was made 20 minutes after the vessels were inserted in the water-bath.

The readings were taken to the nearest 0.5 mm. The average change in the manometer level was about 8 mm. between two readings in the oxygen consumption experiments. The average error of the single determination from this source alone may, therefore, have been as high as $\pm$ 6 per cent, or even higher, if a similar error for the thermobarometer was added. In the determination of the R.Q., obviously an accumulation of errors occurs; for example, the use of different batches of animals for the determination of the oxygen consumption and carbon dioxide production in those series in which Warburg's indirect method was employed might result in reading errors on three manometers (two for the animals and one for the thermobarometer) besides the biological variation. The exact limits of error of the R. Q. determinations are difficult to evaluate. The "extremes" given in the tables doubtless do not represent the true range of biological variability, but are a summation of this and the errors. The conclusions drawn in this paper are, therefore, based on mean values of a number of experiments for each series, as indicated in the tables. The similarities of the mean values of series conducted under comparable conditions are regarded as sufficient proof that the errors inherent in the single determinations have largely been eliminated and that large differences in the mean values of experiments conducted under different conditions are real.

Results

It was noticed in experiments conducted in another connection that the worms showed in vitro a surprising resistance against changes in the osmotic pressure of their surroundings. It seemed therefore of interest

to study the oxygen consumption in various NaCl concentrations (Table I). The solutions used varied from distilled water to 4 per cent NaCl and with each solution seven individual experiments were performed, each lasting four hours. The initial mean values were fairly similar in all series. They were always somewhat higher than those found in the later stages of the experiments, an observation that will be discussed below. It is apparent that beginning with the third or fourth half-hour period a steady stage was reached in the lower concentrations (0–1.5 per cent NaCl), whereas in the higher concentrations (2.0–4.0 per cent NaCl) a more or less continuous decline occurred. The values of the different series belonging to these two groups have been averaged and the resulting curves are shown in Fig. 1. The steady decline of the O_2 consumption in the higher salt concentrations is obviously an indica-

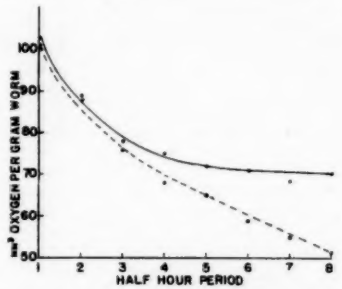

FIG. 1. Oxygen consumption in salines of low and high concentration, mean values. Solid line: 0 to 1.5 per cent NaCl. Broken line: 2 per cent to 4 per cent NaCl.

tion that they are definitely toxic. The fact, however, that variations between 0 and 1.5 per cent NaCl seem not to interfere with the normal respiratory activities, is of interest. This opens up the possibility of studying in future experiments the influence of various substances upon the oxygen consumption irrespective of their osmotic properties.

The fact that these parasites are able to maintain a constant level of metabolism in media of different osmotic pressure has probably a biological basis. Although the life cycle of this species is not known in detail, it can be assumed that the worms live at various stages in surroundings differing, perhaps widely, in molecular concentration.

It should be noted that the variations in the rate of oxygen consumption between different experiments, as indicated by the "extremes" of Table I, were fairly large. Even in the highest salt concentrations, however, some worms were able to maintain a high and fairly regular rate of oxygen consumption. The surprising tolerance of single speci-

mens to a great variety of different media, as judged by their survival in vitro, was frequently noticed and will be discussed in a future paper.

The average oxygen consumption of normal worms was about 140 cu. mm. per gram per hour. It was therefore about twice as great as that reported for *Ascaris* (von Brand, 1934; Harwood and Brown, 1934; Krüger, 1936), but considerably lower than those found in *Setaria equina* (Toryu, 1934) or the hookworm (Harwood and Brown, 1934).

TABLE I

Oxygen consumption of freshly isolated worms kept in salines of different concentrations. Oxygen in cu. mm. per gram of worm. Each mean value is derived from 7 determinations.

Half-hour periods	1	2	3	4	5	6	7	8
0% NaCl								
Mean	94	95	75	83	70	68	71	70
Extremes	51–127	73–112	56–94	56–103	56–88	37–84	55–79	55–83
0.5% NaCl								
Mean	99	80	72	71	73	63	72	78
Extremes	59–130	56–117	40–104	32–123	32–117	21–136	40–143	32–149
1.0% NaCl								
Mean	117	92	81	78	77	80	66	70
Extremes	99–165	46–172	46–178	41–148	48–118	46–137	41–90	40–92
1.5% NaCl								
Mean	103	87	84	67	69	71	64	63
Extremes	63–148	63–134	45–118	28–90	36–92	36–104	27–85	27–89
2.0% NaCl								
Mean	92	82	82	60	60	60	51	55
Extremes	53–161	63–114	39–104	47–82	49–71	49–67	29–65	49–62
3.0% NaCl								
Mean	105	87	86	82	61	63	59	51
Extremes	74–170	60–119	60–112	34–134	17–112	17–100	17–100	17–99
4.0% NaCl								
Mean	105	97	91	61	73	54	56	46
Extremes	83–133	77–109	39–117	19–107	19–115	7–80	14–91	7–83

Unfortunately, a comparison with Fenwick's (1938) data for *Ascaris* larvae is impossible, since he bases his figures on numbers of larvae instead of their weight. Stannard, McCoy, and Latchford (1938) found in *Trichinella* larvae an oxygen consumption of 1.70 cu. mm. per hr. per milligram of dry weight. A calculation of the above *Eustrongylides* figures on this basis, using the previously found figure of 25 per cent dry weight (von Brand, 1938), yields a value of 0.56 cu. mm. Krüger (1940) showed that the rate of oxygen consumption of *Ascaris lumbri-*

coides of various sizes depends rather on surface than on weight. In Table II an analysis of the oxygen consumption of *Trichinella* larvae, *Eustrongylides* larvae and adult females of *Ascaris lumbricoides* has been attempted on this basis. It should be noted that no accurate figures for the fresh weight of *Trichinella* larvae are available in the literature, only data on the dry weight. The fresh weight has been calculated on the basis of 5 per cent dry weight. The conclusion drawn below remains, however, unchanged, even if the weight of the *Trichinella* larvae is calculated on the assumption of 15 per cent dry weight, a figure which is not very likely for these delicate worms. It appears that, contrary to Krüger's findings on individuals of one species, the oxygen consumption of nematodes of very different size is not constant if calculated on the basis

TABLE II

Calculation of oxygen consumption of various helminths on the basis of weight and relative surface.

Species	Weight of 1 worm in mgm.	Relative weight	Surface (Weight $^{2}/_{3}$)	Relative surface	O_2 in cu. mm. per worm per hour	Relative O_2 consumption
Trichinella larva (3)	0.007	1	0.037	1	0.0006	1
Eustrongylides larva (4)	82	11700	18.9	511	12.6	21000
Adult Ascaris lumbricoides, female (5)	4780	683000	284	7700	287	478000

[3] The data of Stannard, McCoy and Latchford (1938) for oxygen consumption in saline have been used. The weight of one worm has been calculated assuming 5 per cent dry weight.

[4] The data of the experiments conducted in 1 per cent saline (Table I) have been used.

[5] The data of von Brand (1934) have been used.

of surface. The difference in the rates of oxygen consumption is considerably smaller, although by no means eliminated if the calculation is based on weight. It is recognized that the data are not complete enough to draw general conclusions, but the nematodes appear to be organisms exceptionally well suited for further work along these lines. Many species of intermediate size are available.

The next series was undertaken in order to ascertain the influence of starvation upon the gaseous metabolism. The worms were kept for 7 days in 1 per cent saline in an incubator at 37° C. and daily determinations of the oxygen uptake and carbon dioxide production were performed. In some instances substitute batches, that had starved a similar period under analogous conditions but for which no initial values were

TABLE III

Oxygen consumption and carbon dioxide production of worms starving in 1 per cent saline at 37° C.

Days starving	0	1	2	3	4	5	6	7
Number of determinations	9	7	8	11	11	13	5	12
O_2 cu. mm./gram/hour								
Mean	152	100	113	104	74	82	85	71
Extremes	121–202	63–120	56–142	75–170	57–99	53–138	62–98	48–95
CO_2 cu. mm./gram/hour								
Mean	158	101	122	113	74	82	85	75
Extremes	115–198	57–119	63–171	86–172	56–93	56–138	69–106	48–118
RQ								
Mean	1.07	1.02	1.06	1.10	1.01	1.01	1.01	1.04
Extremes	0.89–1.28	0.89–1.09	0.82–1.18	0.98–1.28	0.77–1.19	0.87–1.11	0.90–1.12	0.83–1.25

available, had to be used during the later days of the starvation period due to the death of worms in some of the original lots. A slow decline in the rate of oxygen consumption and carbon dioxide production occurred under the conditions of this experiment. The respiratory quotient was found to be rather variable, if individual experiments are considered. The mean values for the single days were, however, rather similar and lay always slightly above 1.0. The mean value for the whole series was 1.04 ± 0.013, and similar mean values were also found in the series mentioned below. They are considerably lower than those reported for *Ascaris* (von Brand, 1934, Krüger, 1937) and also somewhat lower than those found in *Trichinella* larvae (Stannard, McCoy and Latchford, 1938). This indicates doubtless that the metabolism of the worm in question is mainly oxidative, but it seems likely that a small amount of anoxidative processes is superimposed on the oxidations.

In an effort to ascertain whether these surmised anoxidative processes are characterized by the production of acids, a similar series of experiments was conducted in saline containing bicarbonate. The worms were kept between determinations in 1 per cent saline at 37° C. as in the preceding series. The figures of Table IV show that the rate of respiration was somewhat higher than that of worms kept in pure saline. The mean respiratory quotient of all determinations in this series was 1.04 ± 0.019, identical with that found in the previous series. An aerobic acid production can therefore not be demonstrated. Stannard, McCoy and Latchford (1938) found no acid production in *Trichinella* larvae kept under aerobic or anaerobic conditions. They assume the production of as yet unidentified, but non-acidic end-products. The *Eustrongylides* larvae, however, produce, as shown previously (von Brand, 1938), considerable amounts of organic acids, if kept under strictly anaerobic conditions. Naturally the question arises, whether there is such a fundamental difference between the accessory anoxidative processes proceeding under aerobic conditions and the anaerobic processes going on, if no oxygen at all is available. The above series seems to point in this direction. I do not think, however, that this is more than a possibility, hardly as yet a probability. It should be kept in mind that the CO_2 liberated by the small amounts of acids expected would not change the RQ very much. It is furthermore entirely possible that the bicarbonate reserves of the body itself are sufficient to allow neutralization of aerobically produced acids, in which case no difference between the RQ's of worms kept in media with or without bicarbonate could be expected.

The next series was designed to ascertain whether an anaerobic period previous to the determinations would have any influence upon the gaseous exchange. Freshly isolated worms were put in glass vials of about 5 cc.

TABLE IV

Oxygen consumption and carbon dioxide production of worms kept in 1 per cent saline + 0.1 per cent sodium bicarbonate at 37° C.

Days starving	0	1	2	3	4	5	6	7
Number of determinations	12	11	10	8	8	11	8	8
O_2 cu. mm./gram/hour								
Mean	149	143	152	129	115	113	110	102
Extremes	106–206	94–193	106–185	97–198	80–157	77–154	45–240	59–184
CO_2 cu. mm./gram/hour								
Mean	158	150	158	144	121	115	111	119
Extremes	103–276	110–201	95–228	96–214	81–188	70–158	62–252	59–357
RQ								
Mean	1.05	1.06	1.03	1.11	1.03	1.00	1.01	1.06
Extremes	0.84–1.38	0.89–1.21	0.89–1.23	0.94–1.35	0.91–1.16	0.74–1.15	0.93–1.11	0.75–1.94

capacity filled with 1 per cent saline of 37° C. These were then closed with a thin slice of cork, taking care to eliminate all air bubbles between the cork and the fluid by puncturing the cork with a needle and pushing it down into the fluid. A layer of mercury was then put on top of the cork, preventing the diffusion of oxygen into the vial. It was then placed for 18 to 20 hours in an incubator at 37° C. The amount of oxygen dissolved in the saline would roughly last three-quarters of an hour to

TABLE V

Gaseous exchange of worms kept previous to the determinations for 16–18 hours under anaerobic conditions ("post-anaerobic animals" in the table) and of freshly isolated worms ("fresh animals" in the table). Only the mean values are given; the variations were of the same order as those shown in Tables I, III and IV. They were somewhat more pronounced in the "post-anaerobic" than in the "fresh" lot. The O_2 series of the "fresh animals" is the same as that given in Table I for 1 per cent saline.

Half-hour periods	1	2	3	4	5	6	7	8	9	10
Number of determinations										
Post-anaerobic animals	10	10	10	10	10	10	10	10	10	6
Fresh animals	8	8	8	8	8	8	8	8	8	8
O_2 cu. mm./gram										
Post-anaerobic animals	219	169	155	172	154	121	112	99	98	79
Fresh animals	117	92	81	78	77	80	66	70	69	74
CO_2 cu. mm./gram										
Post-anaerobic animals	164	89	96	127	122	133	127	118	107	82
Fresh animals	107	87	86	80	85	82	72	73	73	82
RQ										
Post-anaerobic animals	0.76	0.53	0.58	0.58	0.68	1.02	1.09	1.11	1.15	0.96
Fresh animals	0.90	0.91	1.04	1.03	1.10	1.04	1.08	1.05	1.04	1.10
Excess O_2 cu. mm./gram										
Post-anaerobic animals	145	95	74	104	77	41	46	29	29	5
Fresh animals	43	18	—	—	—	—	—	—	—	—
CO_2 retained cu. mm./gram										
Post-anaerobic animals	68	90	68	55	41	—	—	—	—	—
Fresh animals	17	10	—	—	—	—	—	—	—	—

one hour. Added to this is an unknown amount of oxygen present in the worm tissues and combined with the haemoglobin. After about one and one-half to two hours, the color of the worms turned to the dull red of reduced haemoglobin; the actual period of anaerobiosis was therefore about 16 to 18 hours. During this time they became invariably immotile, but they began to move again shortly after restoration of aerobic conditions. Immediately after the end of the anaerobic period, the worms were placed in the respiratory vessels (Table V,

post-anaerobic animals). The rate of oxygen consumption was very high in the beginning, if compared to that of worms taken freshly from fish (Table V, fresh animals), but then went slowly down, and after 5 hours reached about the normal level. The subtraction of the oxygen values of freshly isolated worms from those of worms previously kept anaerobically reveals a total excess oxygen consumption of 584 cu. mm. per gram. To this figure must be added 61 cu. mm. of excess oxygen found in freshly isolated worms (see below), summing up to 645 cu. mm. This is probably a minimum figure which should be raised for the 20-minute equilibration period for which no data were obtainable. Assuming the same or a slightly higher excess consumption as that found in the first half-hour period, the total excess oxygen can be assumed to lie between 750 and 800 cu. mm. per gram. This excess oxygen consumption can be interpreted in two ways. It could be a purely physical process, i.e. the amount of oxygen needed to restore an equilibrium between the liquids (haemoglobin!) and tissues of the body with the surrounding fluid. In this case the process would not have much significance. The fact, however, that the bright red color of oxyhaemoglobin reappears very quickly after restoration of aerobic conditions indicates a rapid diffusion of oxygen into the body cavity. It is probable that the 20-minute equilibration period, before the first reading was taken, was sufficient to eliminate this factor.

It seems more likely that the excess oxygen consumption represents the repayment of an oxygen debt. The oxygen missed in 16 to 18 hours of anaerobiosis amounts to 2200 to 2500 cu. mm. per gram. The observed excess oxygen consumption of 750 to 800 cu. mm. represents therefore repayment of roughly 30 per cent. Obviously, then, a large part of the oxygen debt was not repaid. The process serves to remove end-products of the anaerobic metabolism. Many free-living organisms are not able to excrete these and they accumulate in the body throughout the anaerobic period. Considerable amounts of oxygen are required during the recovery period to remove them entirely, either by partial resynthesis to glycogen or by oxidation. In frog muscle, for example, a 70 per cent repayment takes place (Rotta and Stannard, 1939) and in insects even much more oxygen is used than was missed, perhaps because a larger percentage of the acids is oxidized (Gilmour, 1940, 1941). It can be safely assumed that in the present case the relatively low percentage of oxygen repayment is correlated with the fact that the worm is able to excrete at least parts of the anaerobic end-products, as evidenced by the fact that the medium becomes markedly acid during anaerobiosis (von Brand, 1938). Obviously, all excreted acids are eliminated from

participation in any recovery process. This can involve only those end-products that are still in the body when aerobic conditions are restored.

The respiratory quotients of the worms in this series were very low during the first 2½ hours, namely 0.63 as compared to 1.06 in the following 2½ hours. In some individual cases exceedingly low values, from 0.04 to 0.08, were observed from ½ to 1½ hours after the beginning of the experiments. These low respiratory quotients represent doubtless a carbon dioxide retention. The amounts of carbon dioxide retained were calculated assuming that the actual respiratory processes also had an RQ of 1.06 during the first 2½ hours. They amounted to a total of 322 cu. mm. per gram.

It is of interest to note that the period of carbon dioxide retention was shorter than that of excess oxygen uptake (2½ against 4½ to 5 hours). The mean R.Q. after the end of the carbon dioxide retention period was, as mentioned above, 1.06,—exactly the same value that was found in the control series with freshly isolated worms (Table V, fresh animals.) This indicates that the recovery processes that are superimposed upon the normal respiratory activities must have very nearly the same RQ as the latter.

It should be noted that so far very little is known about similar processes in parasitic worms. The adult *Ascaris,* which is primarily an anaerobic living organism (von Brand, 1938*a*) shows no accumulation of an oxygen debt during anaerobiosis (Adam, 1932; Krüger, 1936). Newly-hatched *Ascaris* larvae, on the other hand, have for a short time a distinctly increased oxygen consumption that has been interpreted as the repayment of an oxygen debt incurred during development inside the egg shell (Fenwick, 1938).

The observations described above seem to open a way to decide whether the worms lead a predominantly aerobic or anaerobic life inside the fish. If the second alternative were true, one should expect that freshly isolated worms would show the same type of oxygen consumption and similar low RQ's as worms previously subjected to anaerobic conditions in vitro. There is very little evidence that such is the case, as evidenced by the experiments summarized in Table V (fresh animals). It is true that the initial oxygen uptake was slightly higher than it was later on, a fact already mentioned above in discussing the experiments summarized in Table I. The mean respiratory quotients of the first two half-hour periods with 0.90 and 0.91 respectively were somewhat lower than that of the later periods which yielded an average of 1.06. On the basis of these figures, an excess oxygen consumption of about 61 cu. mm. per gram and a carbon dioxide retention of about 27 cu. mm. can be assumed. These are very small

figures if one considers the fact that previous to the determinations the worms had doubtless lived for months in their cysts. One would have expected the repayment of a maximal debt if the oxygen supply had been restricted noticeably. This series seems therefore to establish a sound basis for the assumption that the worms are able to satisfy very nearly their entire oxygen need inside the fish. In accordance with this view is the fact that freshly isolated worms show the bright red color of oxyhaemoglobin.

Summary

1. Variations between 0 and 1.5 per cent NaCl in the medium had no influence upon the oxygen uptake of a larval *Eustrongylides*, but concentrations between 2 and 4 per cent were definitely toxic.

2. The oxygen uptake (140 cu. mm. per gram per hour) was greater than that found in *Ascaris*, but smaller than that reported for *Setaria*, hookworm or *Trichinella* larvae.

3. The oxygen uptake of nematode species of very different sizes remains more constant if calculated on the basis of weight rather than surface.

4. During a week's starvation at 37° C. the oxygen consumption decreased to about half the initial value.

5. The mean respiratory quotient of aerobically kept worms was in all series slightly above one, but no aerobic excretion of organic acids could be demonstrated with the methods employed.

6. In an aerobic period following an anaerobic period of 16 to 18 hours duration, the worms repaid about 30 per cent of the incurred oxygen debt and retained a considerable amount of carbon dioxide.

7. Freshly isolated worms, on the other hand, showed only a trace of excess oxygen consumption and carbon dioxide retainment. It is therefore concluded that the animals inside their cysts in the fish lead an almost purely oxidative life.

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THE INFLUENCE OF SURFACE ANGLE AND OF LIGHT ON THE ATTACHMENT OF BARNACLES AND OTHER SEDENTARY ORGANISMS

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Introduction

In 1935, Hopkins was able to demonstrate that more than six times as many larvae of the Pacific Coast oyster, *Ostrea lurida*, became attached to the under surface of glass plates held in a horizontal position than on the corresponding surfaces of plates at 45°, and about one hundred times as many as became attached to plates in the vertical position. The practical significance of these results to oyster growers is now well known.

The present study was undertaken to determine the behavior of other sedentary organisms in relation to plane glass surfaces held at various angles. The identification of the species involved, the number of individuals attached, and the success of colonial types, as measured by the size of the colony, could be established with precision by the use of such plates. It was hoped that, in addition to its ecological value, this study might contribute to the general problem of ship fouling.

Materials and General Methods

Three pairs of window glass 6 by 10 inches which had been sandblasted on both sides to give a regular but roughened surface were bound two inches apart by an encircling band of one-half inch mesh galvanized hardware cloth. Three pairs of such units were then wired to the floor and sides of a heavy wire cage which could be lowered into the sea. One pair of plate units was arranged in a horizontal position, another in a vertical, and a third at an angle of 45° to the horizontal. Considering both sides, each series of 18 plates offered surfaces at five different angles; 0° (under horizontal), 45° (under 45° plate), 90° (vertical plate—both sides), 135° (upper side of 45° plate), 180° (upper side of horizontal plate). In such series a total of 360 square inches of surface was available for the attachment of sedentary organisms at each angle excepting at 90°, where twice this amount was present. The values at this angle, therefore, were regularly halved in the presenting of data in this study.

Series of plates were placed in the sea at two points close to the Bureau of Fisheries Laboratory at Pensacola, Florida. Location 1 was the *north dock* of the island facing the open water of Santa Rosa Sound. This station was characterized by an active current and wave action. The plates were kept at a mean level of 9 feet below the surface and this was about 2 feet above the bottom. Location 2 was the *boat slip* at the southwestern end of the island. Here the current was mild and the wave action generally slight. The heavy wire cages containing the units of plates were placed directly on the sea floor so that the lowest plates were 8 inches from the bottom and the top plates were covered by about a foot of water at low tide.

After various periods of residence in the sea, plates were washed gently with fresh water to remove excess silt and salt and then dried. Counts of the organisms attached were made with the aid of a dissecting microscope.

Identifications of the Bryozoa were made by Dr. Raymond C. Osburn of Ohio State University, to whom the authors wish to express their thanks.

The set-up and the technique followed in this study were essentially those devised by Dr. A. E. Hopkins. We are deeply indebted for his friendly assistance and suggestions. Appreciation is also acknowledged to the Bureau of Fisheries for the use of laboratory facilities at Pensacola, Florida.

Results Obtained for Acanthodesia tenuis and Electra hastingseae

Sand-blasted plates maintained at the *boat slip* station accumulated relatively few barnacles but were characterized by a heavy growth of Bryozoa, particularly the encrusting species *Acanthodesia tenuis* and *Electra hastingseae* (see Tables I and II). Counts were made of the individual number of colonies for each species attached to the glass surfaces held at the various angles. Colonies of *A. tenuis* were always more numerous than *E. hastingseae* and the size of populations was always greater for plates kept in the sea for the longer period. More than 90 per cent of all Bryozoa studied were found attached to plates held at 0° and 45°. This was true for both time intervals studied. At 0° practically no portion of the plate remained uncovered after 37 days exposure.

In addition to counting the number of individual colonies, an attempt was made to measure the relative "success" of the attached larvae in relation to the angle of orientation. Planimeter measurements were

TABLE I

Twenty-three Day Plates (5/12/40 to 6/4/40)
Number of individuals and area in square centimeters of bryozoan colonies attached to sand-blasted plates in boat slip.

		A. tenuis			*E. hastingsae*					
		Number of colonies	Total area of colonies	Average area of colonies	Number of colonies	Total area of colonies	Average area of colonies	Barnacles	Bivalves	Hydroids
0°	1.	74	245.7	3.32	20	19.3	0.97	47	29	4
	2.	91	297.5	3.27	12	15.6	1.30	170	136	7
		165	543.2	3.29	32	34.9	1.09	217	165	11
45°	1.	59	208.9	3.54	25	16.7	0.67	9	6	2
	2.	66	129.2	1.96	26	15.9	0.61	14	20	2
		125	338.1	2.71	51	32.6	0.64	23	26	4
90°	1.	4	1.1	0.28	0	0.0	0.00	1	0	0
	2.	5	1.2	0.24	1	0.2	0.20	4	0	1
		9	2.3	0.26	1	0.2	0.20	5	0	1
90°	1.	2	0.2	0.10	2	0.4	0.20	14	1	0
	2.	3	1.2	0.40	2	1.0	0.50	19	1	1
		5	1.4	0.28	4	1.4	0.35	33	2	1
135°	1.	3	1.1	0.37	1	0.1	0.10	1	0	0
	2.	0	0.0	0.00	2	0.2	0.10	3	0	1
		3	1.1	0.37	3	0.3	0.10	4	0	1
180°	1.	5	0.6	0.12	0	0.0	0.00	1	1	0
	2.	1	0.1	0.10	0	0.0	0.00	4	1	2
		6	0.7	0.12	0	0.0	0.00	5	2	2

therefore taken at the margin of each colony. A minor source of error introduced as a result of occasional over-riding edges was further complicated by the physical limitation for the spread of individuals on plates at 0° due to the density of the population. The total area and the average area of the two species of Bryozoa under discussion yielded results which resembled those found for the relation between the number of colonies and the angular orientation. That is, not only were there more individual colonies at 0° and 45°, but the ancestrulae attached on plates in these positions were more successful in giving rise to zooids.

TABLE II

Thirty-seven Day Plates (4/28/40 to 6/4/40)
Number of individuals and area in square centimeters of bryozoan colonies attached to sand-blasted plates in boat slip.

		A. tenuis			*E. hastingseae*					
		Number of colonies	Total area of colonies	Average area of colonies	Number of colonies	Total area of colonies	Average area of colonies	Barnacles	Bivalves	Hydroids
0°	1.	104	752.0	7.23	37	97.0	2.62	45	15	5
	2.	106	795.0	7.50	45	72.7	1.62	171	25	0
		210	1547.0	7.37	82	169.7	2.07	216	40	5
45°	1.	62	585.9	9.45	23	40.1	1.74	9	8	1
	2.	61	377.1	6.18	48	71.9	1.50	16	2	0
		123	963.0	7.83	71	112.0	1.58	25	10	1
90°	1.	5	19.3	3.86	2	9.6	4.80	2	0	0
	2.	9	14.5	1.61	6	1.0	0.17	0	0	0
		14	33.8	2.41	8	10.6	1.33	2	0	0
90°	1.	3	8.0	2.67	4	4.6	1.15	4	0	0
	2.	7	17.9	2.56	1	2.5	2.50	7	0	1
		10	25.9	2.59	5	7.1	1.42	11	0	1
135°	1.	2	0.4	0.20	0	0.0	0.00	6	0	0
	2.	4	1.2	0.30	0	0.0	0.00	14	0	0
		6	1.6	0.27	0	0.0	0.00	20	0	0
180°	1.	0	0.0	0.00	0	0.0	0.00	0	0	0
	2.	2	0.3	0.15	0	0.0	0.00	0	0	0
		2	0.3	0.15	0	0.0	0.00	0	0	0

POPULATIONS ON PLATES AT KNOWN ANGLES EXPOSED FOR FIFTEEN DAYS

Counts were made of seven groups of organisms which had become attached to a series of plates held at 0°, 45°, 90°, 135°, and 180° placed at the *north dock* for a period of 15 days (5/31/40 to 6/15/40); 19,751 individuals were found for the total area of 1800 square inches (see Table III).

Characteristically, the position designated as 0° (under surface of plates in the horizontal position) and 45° supported the largest number of individuals of all types with the exception of the ivory barnacle,

Balanus eburneus. The behavior of this organism will be discussed in another section of this study. In contrast to plates exposed in the *boat slip* for 23 days and for 37 days during approximately the same time, the 15-day plates at the *north dock* carried a total population which was more than twice as large. Colonies of *A. tenuis* though very numerous were made up of very few individuals. Plates at 0° were so densely covered with organisms that the counts reported probably represent the maximal limits of populations possible under conditions of extreme overcrowding. Coe (1932) has noted that numerous young may seriously interfere with early settlers by depriving the latter of food and oxygen.

Colonies of *E. hastingseae* were not numerous although it must be stated that identification of recently attached Bryozoa is difficult and

TABLE III

Fifteen-Day Series (5/31/40 to 6/15/40)
Distribution of 19,751 organisms attached to sand-blasted glass plates at *north dock*.

	Barnacles	*Acanthodesia tenuis*	*Electra hastingseae*	*Bugula neritina*	Bivalves	Hydroides sp.	Sponge
0°	1,630	1,881	18	299	3,178	25	25
45°	3,846	1,713	16	194	703	10	26
90°	1,125	344	0	35	246	4	0
135°	1,863	102	0	18	271	2	0
180°	2,029	37	0	1	110	0	0
Total	10,493	4,077	34	547	4,508	41	51

that some may have been mistaken for *A. tenuis*. *Bugula neritina* grew luxuriantly.

The bivalves were principally oysters, although there were some other species present. The results obtained for this group closely resemble those reported by Hopkins (1935) for the Olympia oyster.

Calcareous tubes of an annelid worm, probably belonging to the genus *Hydroides*, and a small white sponge were counted on the various plates. Taxonomic work on both of these organisms is still in progress.

SPECIAL PROBLEMS INVOLVED IN THE STUDY OF BARNACLES

Counts made of the number of cypris larvae and of metmorphosed barnacles have shown no consistent and regular tendency to follow the general pattern observed for other animals reported in this study; namely, that there is a gradual falling off in the numbers of sedentary organisms as the angle of plane surface is increased to 180° (upper horizontal surface). Instead, there is a considerable irregularity in the

relations between population density and the angle of orientation (Tables I, II, III and IV). Values did tend, however, to be elevated at 0 and 45° but depressed at 90°. It was suspected that light might be the primary complicating factor and experiments were therefore designed to test its possible influence on the geotropic response.

In addition to light, other possible variables responsible for the behavior of barnacles under conditions of the experiments were believed to include overcrowding, competition with other organisms, and the influence of primary surface films. In order to test these three variables, very short periods of exposure were tried with special types of plates.

TABLE IV

Numbers of barnacles attached to sand-blasted plates held at various angles

	24-Hour (6/5–6/6)				24-Hour (6/11–6/12)		
	Cyp.	Ad.	T.		Cyp.	Ad.	T.
0°	1,370	99	1,469	0°	163	630	793
45°	961	303	1,264	45°	243	854	997
90°	748	181	*929	90°	94	463	557
135°	259	253	512	135°	234	1,057	1,281
180°	129	74	203	180°	253	1,147	1,400
Total	3,467	910	4,377	Total	987	4,151	5,028
	5-Day (5/31–6/5)				15-Day (5/31–6/15)		
	Cyp.	Ad.	T.		Cyp.	Ad.	T.
0°	976	2,024	3,000	0°	369	1,261	1,630
45°	554	2,155	2,709	45°	533	3,313	3,846
90°	603	3,285	*3,888	90°	42	1,083	1,125
135°	73	2,443	2,516	135°	25	1,838	1,863
180°	42	2,373	2,415	180°	35	1,994	2,029
Total	2,248	12,280	14,528	Total	1,004	9,489	10,493

* double plates.

Fortunately, the plankton was exceedingly rich in the experimental area during June, 1940, so that the heavy sets of cypris larvae needed for statistically significant analysis could be obtained with very short periods of immersion.

Black, clear and opal glass plates of the type commonly employed as mounting elements in museum jars were selected for these experiments. These plates present smooth surfaces and are useful in testing photic responses without the chemical complications arising when paints are used. All plates measured one and seven-eighths inches by seven and one-quarter inches. Pairs were held in an encircling band of quarter-inch mesh galvanized hardware cloth. Such units offered a total of

approximately 27.2 square inches of surface for each pair of sides. Plates were lowered into Santa Rosa Sound at the end of the *north dock* of the Bureau of Fisheries Laboratory. The mean depth at which they were placed was 8 feet, this being about 3 feet above the bottom. A window weight was used as ballast for each unit. At various intervals plates were removed, washed gently with fresh water and allowed to dry. Counts for both cypris larvae and metamorphosed adults of the barnacle *Balanus eburneus* were made at magnifications of from 6 to 30 times.

While provisional 24-hour experiments with sand-blasted plates (Table IV) had already shown that populations of *B. eburneus* at 180°

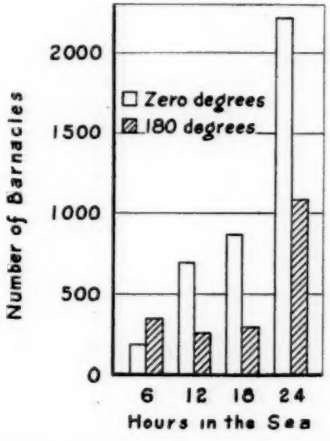

FIG. 1. Barnacle populations on the upper and lower surfaces of plates held in a horizontal position.

were not regularly much lower than those at 0°, these results were confirmed and extended with the use of black plates on whose surface cypris larvae and young adults could be easily counted. An example of this type of test in which 4 sets of plates held in a horizontal position were examined respectively at the end of 6, 12, 18, and 24 hours is shown in Fig. 1. While the increase in numbers of larvae at 0° was regular with time, this was not true for the surface designated as 180°. There were relatively more barnacles on this surface at 6 hours, and by the end of 24 hours the number on the 180° side was about half as great as that found for 0°.

In another series of experiments conducted for 24 hours (Fig. 2) using black, opal and clear plates held both in the horizontal and in the vertical positions, the black plates consistently showed much larger populations. In addition, the variable nature of the response to the geotropic factor was here again confirmed. For all three types of plates the lowest

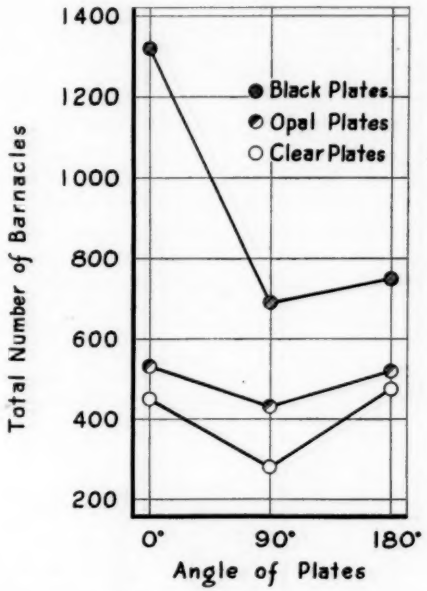

FIG. 2. Orientation of barnacles on black, opal and clear plates held in the horizontal and vertical positions.

counts were found at the 90° angle. The total number of individuals at the three angles were:

Black	2750
Opal	1484
Clear	1204

Table V shows the results of an experiment in which one unit of black and one of clear plates were examined after being held in a horizontal position in the sea for periods of 1, 3, 5, and 7 days. The results show: (*a*) no significant difference in the population size of the under

TABLE V

Barnacle populations on clear and black plates after exposure for one, three, five and seven days.

Days	Clear			Black		
	0	180	Total	0	180	Total
1	217	287	504	1,514	1,113	2,627
3	851	844	1,695	2,325	2,022	4,347
5	1,006	630	1,636	2,174	2,508	4,682
7	906	863	1,769	1,774	2,303	4,077
	2,980	2,624	5,604	7,787	7,946	15,733

as compared to the upper plane surface; (*b*) individual and total numbers of barnacles for the various periods were always more than twice as great on black plates; (*c*) units which remained in the sea 5 and 7 days did not accumulate populations significantly larger than those on the 3-day plates. This last observation suggests that some limiting factor was in operation. If this had been due to the accumulation of silt, its effect should have been most evident on the upper surfaces of plates (180°). A more probable hypothesis is that a slime film which may have formed within the first 5 days became a limiting condition. The fact

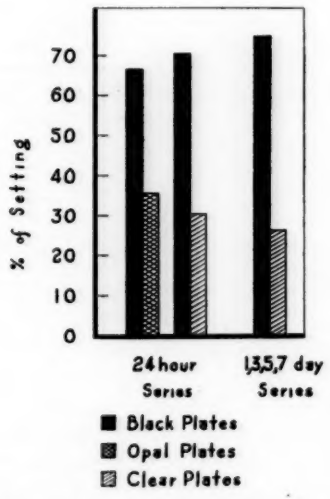

FIG. 3. Percentage of barnacles attached to black *versus* opal and clear plates.

that a plateau in the population was reached at approximately 1700 individuals on the clear plates, whereas the population on the black plates exposed under identical conditions reached a level of about 4400 individuals, gives evidence that the phenomenon was not due to overcrowding. Bryozoa, bivalves, and sponges were still too few by the end of the seventh day to have been limiting factors. Visscher (1927), quoting Hillen and Angst (1923), has signaled the controversy concerning the significance of primary films and has pointed out the need for further studies of this factor in relation to the attachment of fouling organisms.

The importance of the photic factor in the attachment of barnacles may be seen in Fig. 3, which is a histogram summarizing the percentage of sets on black versus clear and opal plates. In these and other similar experiments, when all other conditions remain constant, twice as many

TABLE VI

Distribution of cypris larvae on pairs of plates offering 27.2 square inches of surface exposed from 9 P.M. to 3 A.M.

		Black			Opal			Clear		
Date	Expt. No.	0°	180°	Total	0°	180°	Total	0°	180°	Total
6/29–30/40	1	62	48	110	42	41	83	29	62	91
"	2	37	13	50	47	53	100	47	53	100
6/30–7/1/40	3	44	39	83	34	65	99	36	72	108
"	4	39	59	98	61	50	111	31	64	95
Totals		182	159	341	184	209	393	143	251	394

barnacles were found attached to black plates as were found on opal or clear plates.

As a method for proving the reality of the photic factor, experiments were conducted on moonless nights between the hours of 9 p.m. and 3 a.m. During these six-hour periods, when visibility was at a minimum, barnacle sets were collected on various types of plates. Population counts were made for two units of plates on two different nights. The total number of organisms on black, opal, or clear plates was remarkably similar (Table VI). Thus it would appear that the outstanding characteristic found in the study of the barnacle, *Balanus eburneus,* that is, the aggregation of notably larger numbers on dark surfaces, must be in the nature of a phototropic response, since the phenomenon does not occur when light is at a minimum.

In his important study on the fouling of ships' bottoms, Visscher (1927) presented evidence showing that barnacles are strongly negative to lights at the time of their attachment. He advocated the quest for substances of light color to serve as an adequate substitute for the red and brown paints which are generally used. The findings of the present study support these conclusions and, in addition, indicate the need for experiments on the effect of illumination in the control of ship fouling.

Summary and Conclusions

1. A method is described for studying the attachment of the larvae of sedentary marine organisms to plane surfaces held at various angles to the horizontal. The use of black, opal and clear glass plates to analyze the photic factor is presented.

2. In the curve describing the attachment of six different groups of organisms in relation to the angle at which plates were held, the largest populations always were found at zero degrees (the under surface of plates held in the horizontal position) and the numbers decreased as the angle increased.

3. Planimeter measurements of the colonial bryozoans, *Acanthodesia tenuis* and *Electra hastingseae,* showed further that the area, and therefore the success of the colonies, was likewise a function of the angle at which the plates were held, the greatest growth being at zero degrees.

4. In contrast, the ivory barnacle, *Balanus eburneus,* was not regularly found to orient to the geotropic factor in the same manner as the other sedentary organisms which were encountered.

5. Short-time experiments proved that the initial orientation of the barnacles probably was not limited by other organisms or by silting, although some evidence was found for the view that a primary film may have limited the attachment between the third and fifth day.

6. Under conditions of natural day-night exposure, twice as many barnacles became attached to black as to opal or clear glass plates. In experiments conducted exclusively at night no difference was observed in the size of populations attached to black, opal or clear plates. This suggests that a photic factor is of primary importance in the attachment of the barnacle, *Balanus eburneus.*

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THE DIFFERENTIATION OF GEOGRAPHICAL GROUPS IN LYMNAEA PALUSTRIS

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Introduction

This paper is a first report on a part of the experimental work on individual and group differences in *Lymnaea palustris*, reared under standardized laboratory conditions during the years 1937 to 1940. The actual experiments were conducted jointly by the two authors. The analysis of the data and their presentation in this paper are primarily the work of the first author, who wishes at this point to express full appreciation of the advice and assistance given by Professor Frederick E. Croxton and Mr. Richard H. Brown in connection with the statistical analysis.

The investigation began with a study of *Lymnaea rubella*, indigenous to the Hawaiian Islands, and was later extended to include the local species, *Lymnaea palustris*, for comparison. Two independent collections of *Palustris* were made, one by the second author from a small stream not far from Croton, New York, and the other by the first author from a mill-pond in Newtown, Connecticut. These groups are herein designated as *Lymnaea palustris* Newtown and *Lymnaea palustris* Croton. It soon became clear that the Newtown snails, though morphologically indistinguishable, differed widely from the Croton snails in their physiological processes of growth, fertility, and longevity. The problem was to determine whether these physiological differences were of sufficient magnitude to be significant characteristics of two distinct geographical groups or races, or whether they were to be regarded as the expression of individual variation within one group or population. The problem is of fundamental importance for the analysis of evolution.

An extensive review of the literature is unnecessary here. The problems presented by geographical races are fully discussed by Robson and Richards (1936), Dobzhansky (1937), and by the collaborators of the recent volume "The New Systematics" edited by Huxley (1940); noteworthy chapters in the last-named are those by Huxley (1940), Muller (1940), and Diver (1940). Diver's (1939) general discussion of racial variation must also be mentioned.

For the most part, morphological characters of diverse local groups have been investigated. As representative studies, we may cite those of Rensch (1929) on birds, Gulick (1905) on Achatinellidae, Bartsch (1920) on *Cerions,* Fischer-Piette (1938) on *Patella,* Crampton (1916, 1925, 1932) on *Partula;* Goldschmidt (1934) on *Lymantria,* and Mozley (1935) on *Lymnaea.*

Dobzhansky (1935) has demonstrated that in *Drosophila pseudo-obscura* there are two races which are morphologically indistinguishable but physiologically unlike. Working with the same material, Shapiro (1932) and Poulson (1934) have likewise shown that physiological differences between two races exist, while Lancefield's investigation (1929), confirmed by Dobzhansky and Boche (1933), proved that the two races can be distinguished also by the differing forms of the Y chromosome. Recently, Baily (1939) has published an important study on " Physiological Group Differentiation in *Lymnaea columella.*"

Our methods of culture of *palustris,* as well as the adopted treatment on the data, differ from those employed by Baily in his work on *columella.* No prior physiological studies on *palustris* are known to us. This species of *Lymnaea* is very different from *columella* and also from *rubella* in its physiological processes of growth and reproduction. It is intermediate between them in the length of its life cycle.

The animals of our experiments were kept in a greenhouse during the winter months in quart jars filled with water to approximately the 500-cc. level. Efforts were made to keep the temperature between 55° and 75° F.; the optimum seemed to be from 60° to 70°. When the temperature grew too warm, toward the end of May, the snails were transferred to cooler laboratories, there to remain until the end of September, when they were returned to the roof greenhouse.

Spring water was used consistently because the animals were adversely affected by the city water. Each jar contained a spray of Cabomba, to provide a substratum for the deposition of eggs, as well as a small amount of finely-sifted earth. The standard food was lettuce of the " Boston " variety, and some of this was always available in the jars.

The individual masses of eggs were removed from the jars soon after they were laid, and were then kept in glass cups of 50-cc. capacity until the young began to hatch. Each cup with its contents was then placed in a standard quart jar where the growing animals passed their early lives in company. Obviously all of the products of a single mass of eggs were kept under identical conditions. When the young snails reached the age of eighty days, they were measured and isolated in the

standard quart jars. Thereafter they were re-measured at eighty-day intervals, and full records of their fertility were secured until they died.

While some of the original wild individuals may have mated before they were collected, the isolated members of the later generations could not have done so. Therefore all of the eggs deposited by the snails of the pedigreed generations were the products of self-fertilization on the part of the animals. As two polar bodies are formed, the possibility of parthenogenesis is excluded. The statistical methods employed in the present study are those which have been developed by Fisher (1938), Mahalanobis (1932), and Snedecor (1934, 1937) for the analysis of variance. When two groups are compared on the basis of measurable characters, the difference between the means may be estimated as significant of a real distinction by the use of the F test. Technically, F is the ratio of the variance between the means to the variance within the separate groups. If the variance between the means is so great as compared with the variance within the separate groups that the variance between the means cannot be regarded as happening by chance, then the hypothesis that the groups have been drawn from the same population must be rejected. It follows then that the two groups show a significant difference, i.e. a difference which is not attributable to chance variation.

In our tables N is the number of individuals in the sample; $\overline{X}$ is the arithmetical mean, and r is the conventional coefficient of correlation; while F is a critical value which, when taken in conjunction with the proper degrees of freedom, can be denoted as significant or not significant by reference to an F table such as that available in "Applied General Statistics" (pp. 878–879) by Croxton and Cowden. The degrees of freedom involved are: between the means, $n_1 = 1$; and within the samples, $n_2 = N_1 - 1 + N_2 - 1$, where $N_1 =$ the number of items in one sample, and $N_2 =$ the number of items in the other sample.

Fertility

The term fertility as used in this report refers to the egg-laying activity or productivity of the snails. It has no reference to the viability of the eggs or of the later embryos and growing young.

The original *Lymnaea palustris* constituting the first, or parent generation was collected in October, 1937. It comprised snails that had hatched during the previous spring or early summer, as judged by the observations on the growth and life cycles of later generations. The animals were large enough and old enough to lay soon after they were brought into the laboratory. Only15 of the 34 Newtown snails and 14 of the 45 Croton snails produced any eggs, whatsoever. In nature these

animals are relatively inactive in winter; we found that no eggs were laid by individuals kept at low temperatures in a cold room.

As shown in Fig. 1, both groups of snails began to lay abundantly during March, and reached the peak of egg production in April. Egg-laying by the Newtown snails then fell off rapidly to a low level in August, after which it was sporadic until December, when the last Newtown snail died at an age of approximately a year and a half. In

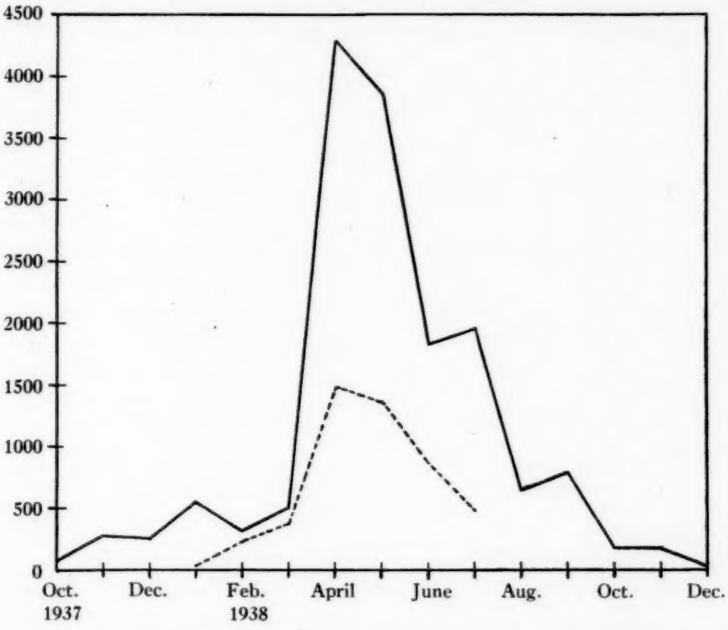

FIG. 1. Egg-laying cycles of the Newtown snails (solid line) and of the Croton snails (broken line) of the parent generation. Y axis—number of eggs produced: 500, 1,000, 1,500, 2,000, 2,500, 3,000, 3,500, 4,000, 4,500. X axis—calendar months: October, 1937 to December, 1938.

contrast, the Croton snails stopped laying quite abruptly in July and they died soon afterward, apparently having reached the end of their normal life cycle, for members of the succeeding generations of this group seldom lived longer than 240 to 320 days. Possibly the heat waves of July hastened their deaths, for it was found later that the Croton snails did not survive extreme hot weather as well as did the Newtown animals.

In comparing the fertilities of the Newtown and Croton groups, several characteristics of reproduction and of the reproductive cycle were

taken into account; namely, the number of egg masses laid, the total number of capsules containing fertile eggs, the number of empty capsules laid, the age of the animal at the onset of fertility, and the length of the fertile period. Table I gives a comparison of the Newtown group with the Croton group in the first, or parent, generation. Each group comprised 15 laying individuals.

The *F* tests show that the Newtown animals were the more productive. They also had a longer fertile period and they laid fewer empty capsules. Further, the Newtown group laid more egg masses than the Croton group, as the means show, but the difference in this respect is not mathematically significant. Colton's statement that the laying of empty capsules comes at the end of the reproductive cycle in *columella*, as a sign

TABLE I

Comparative fertility of the Newtown and Croton snails of the parent generation

Character	*N*	$\bar{X}$	*F*	Significance
Number of egg masses				
Newtown	15	54.4		
Croton	15	32.0		
			3.332	none
Number of eggs laid				
Newtown	15	1049.80		
Croton	15	320.00		
			9.334	positive
Number of empty capsules				
Newtown	15	5.33		
Croton	15	22.73		
			6.105	positive
Length of fertile period in days				
Newtown	15	122.27		
Croton	15	62.27		
			5.486	positive

of decadence, is borne out by our experience with that species, but in *palustris* this does not seem to be equally true.

Egg production in both groups was ascertained for successive 10-day periods for each snail. In each series the peak of production came in the first or the second 10-day period after the onset of fertility. During the first 20 days of laying, the Croton snails produced 968 eggs in 71 egg masses; the Newtown snails laid 2506 eggs in 91 egg masses. After the peak, production fell off gradually until the 151–160-day period for the Croton group and the 281–290-day period for the Newtown series, and soon afterwards the last survivors died.

The second generation, so-called, comprises the offspring of the original or parent animals. The series described by the figures of Table

II were selected from families whose reproductive cycles were approximately synchronized as to the season of the year. In addition, other calculations were made using random samples of all of the families of this generation and the results are given in Table III. Clearly the group differences that appeared in the first generation were consistently repeated in the second generation, as regards the total number of eggs, the number of empty capsules, and the length of the fertile period. In addition, there was a marked difference between the two series in the age at onset of fertility; here there is no comparison to be drawn with the parent generation, since the ages of the latter were only approximately known.

TABLE II

Comparative fertility of the Newtown and Croton snails of the second generation

Character	N	$\bar{X}$	F	Significance
Number of egg masses				
Newtown	24	49.96		
Croton	24	19.25		
			12.265	positive
Number of eggs laid				
Newtown	24	786.33		
Croton	24	132.50		
			11.706	positive
Number of empty capsules				
Newtown	24	8.25		
Croton	24	34.46		
			8.835	positive
Age at onset of fertility in days				
Newtown	19	180.63		
Croton	47	97.38		
			122.621	positive
Length of fertile period in days				
Newtown	24	98.33		
Croton	24	40.79		
			13.430	positive

The possibility of a seasonal variable in the fertility of *palustris* must be taken into account. As already noted (Fig. 1), the members of the parent generation laid few eggs during the autumn and winter and it was not until spring that they began to produce abundantly. Because the reproductive cycle of the Newtown snails differed so much from that of the Croton animals in succeeding generations, it was not possible to demonstrate a seasonal variable in their fertilities. The families compared in Table II came from eggs laid at about the same time of the year, but the Croton snails generally began to lay at earlier ages than the

Newtown snails, and they had a shorter laying span, thus making the exact synchronization of their laying records impossible. The longest laying period of the Croton group was 90 days while that of the Newtown snails was 240 days. Both groups reached the peak of production during the first two 10-day periods of their laying cycles, as in the parent generation. The Croton group actually produced more eggs during the first 20 days, but they ceased to lay much earlier than did the Newtown animals, whose total productivity was much greater in the end.

From our observations, it seems that under laboratory conditions the seasonal rhythm of laying tends to disappear, and that the onset of fertility occurs when the snails reach a certain size, regardless of the time of the year. The significant point is that the characteristic differences between the Newtown and the Croton groups of the parent generation, as regards the features of the productive cycle, were repeated in the second as well as in the later generations reared in the laboratory.

TABLE III

Comparative fertility of random samples of all Newtown and Croton snails of the second generation

Character	N	$\bar{X}$	F	Significance
Number of egg masses				
Newtown	80	39.71		
Croton	63	17.22		
			24.731	positive
Number of eggs laid				
Newtown	80	506.37		
Croton	63	139.36		
			16.192	positive
Number of empty capsules				
Newtown	80	7.76		
Croton	63	24.41		
			23.151	positive

It was impossible to procure full fertility records for all of the families of the third generation, owing to the abundance of the material. The comparison here made employs the two most productive and long-lived families of the contrasted groups (Table IV). The results of the F tests on various aspects of fertility show that the Newtown snails were again more productive than the Croton snails. While the differences are not positively significant, the animals of the former group laid more egg masses throughout a longer period of time, and their ages at the onset of fertility were greater.

Passing to the fourth generation, the Newtown group comprised the relatively large number of 278 snails; the Croton group was made up of 132 individuals belonging to five families, of which only three lived long enough to provide data for complete life cycles. Representative Newtown and Croton families are compared in Table V. Unfortunately, the number of satisfactory Croton layers is small; in fact, the Croton animals proved to be so far inferior to the Newtown snails in their fertility that their breeding had to be discontinued after this generation. The Newtown series, on the other hand, have continued to flourish. They were so weeded out that the stock of later generations has been made up of the descendants of only one of the original snails.

TABLE IV

Comparative fertility of the Newtown and Croton snails of the third generation

Character	N	$\bar{X}$	F	Significance
Number of egg masses				
Newtown	21	24.67		
Croton	12	18.17		
			1.697	none
Number of eggs laid				
Newtown	21	402.81		
Croton	12	238.83		
			4.677	positive
Number of empty capsules				
Newtown	21	2.71		
Croton	12	16.00		
			8.472	positive
Age at onset of fertility in days				
Newtown	21	133.51		
Croton	12	110.25		
			3.218	none
Length of fertile period in days				
Newtown	21	68.30		
Croton	12	52.33		
			1.328	none

The data of Table V are self-sufficient. In this generation it is again clear that the Newtown snails differed from the Croton snails in their actual productivity, and in the same ways as in the earlier series. As the physiological differences in question are exhibited by the two contrasted series throughout four generations, there can be little doubt that the Newtown and Croton groups constitute two geographical races which are presumably unlike in some particulars of their genetic constitutions.

In concluding this section, a partial record is given of the correlations displayed by the several features of fertility with one another and

TABLE V

Comparative fertility of the Newtown and Croton snails of the fourth generation

Character	N	$\bar{X}$	F	Significance
Number of egg masses				
Newtown	27	56.15		
Croton	7	6.71		
			19.615	positive
Number of eggs laid				
Newtown	26	614.46		
Croton	7	99.43		
			16.042	positive
Number of empty capsules				
Newtown	26	4.61		
Croton	7	9.86		
			3.436	none
Age at onset of fertility in days				
Newtown	26	180.77		
Croton	7	120.43		
			24.471	positive
Length of fertile period in days				
Newtown	26	123.96		
Croton	7	12.28		
			22.954	positive

with additional characters. The items of Table VI are representative of numerous calculations of data relating to many series.

As would be expected, productivity as indicated in terms of the number of egg masses and of the number of fertile eggs is positively correlated with the length of the fertile period. The total number of eggs laid and the length of the fertile period are correlated with age at death to significant degrees. It is otherwise with the age at onset of fertility, for this feature does not show a definite correlation either with total productivity or with age at death.

TABLE VI

Correlations in three Newtown families of the second generation taken as random samples

Characters	N	r	F	Significance
Number of egg masses and length of fertile period	24	.901	94.897	positive
Number of eggs and length of fertile period	24	.743	27.107	positive
Number of eggs and age at death	40	.532	15.016	positive
Length of fertile period and age at death	40	.806	70.447	positive
Age at onset of fertility and total productivity	40	.258	2.719	none
Age at onset of fertility and age at death	40	.177	1.229	none

Growth

The main purpose of this section is to compare the Newtown and Croton groups of *palustris* on the basis of the mode of growth and of size at certain stages in the life cycles of the snails. It will appear that differences in these characteristics are quite as definite as in the physiological attributes discussed under the head of fertility.

It will be recalled that the young snails hatched from a single mass of eggs were reared in one and the same nursery jar until they were 80 days old, at which time they were measured and isolated in individual

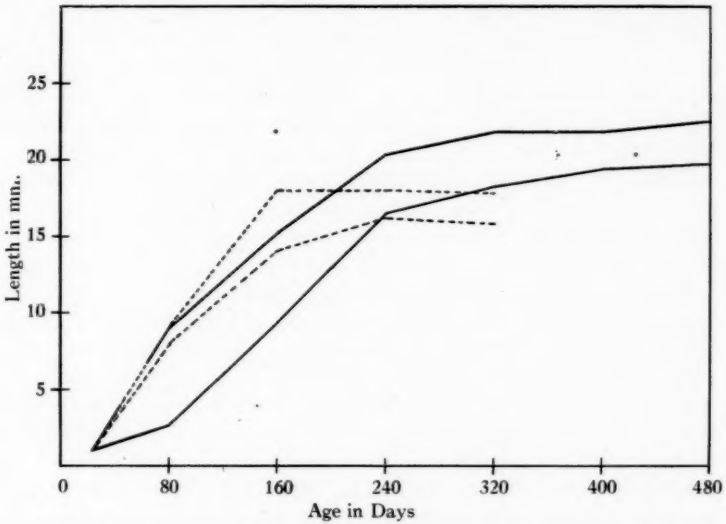

Fig. 2. Growth curves of the fastest-growing and the slowest-growing snails of a typical Newtown family (solid line) and of a typical Croton family (broken line). Y axis—length in mm.: 5, 10, 15, 20, 25. X axis—age in days: 80, 160, 240, 320, 400, 480.

quart jars, thereafter to be measured at 80-day intervals. The total length of the shell was taken as the index of size; measurements of width were also made, but they have not been employed in the present study. In Baily's work on *columella* (1939), the length of the aperture was used as an index of size. Baily measured his animals every five days and thus obtained enough data to plot a logistic curve of growth for each of his two comparable groups. Merrell (1931) investigated the growth of rabbits and plotted a similar logistic curve for each individual and for the whole series of observed animals. Our own meas-

urements are relatively few, but they describe the animals in definite terms at actual successive age-periods in their several cycles. Whatever their limitations, they provide a concrete basis for the determination of group averages and for an estimate of the significance of group differences by the use of the *F* test. The results are given in Fig. 2 and in Tables VIII to XI.

The length of approximately 1 mm. at about 20 days is taken as the starting point of the curves of growth, even though variations are found at this time and even earlier, just as they are throughout the entire life cycle. During the first hour after a mass of eggs is deposited, water is absorbed by the gelatinous matrix, by the individual egg-capsules, and by the actual eggs themselves, with some degree of enlargement as a consequence. The eggs remain the same in size after the first hour until they begin to divide, about three hours after deposition. The diameter of the eggs of one representative series of *palustris* ranged from 0.108 mm. to 0.126 mm., with an average of 0.115 mm. and a coefficient of variation of 5.36. The early embryos vary considerably even before they reach the time of hatching, and there are also considerable differences between broods as regards the amplitude of variation before emergence. In one Newtown series numbering 30, measured at 13 days, the length ranged from 0.882 mm. to 1.008 mm., the average was 0.952 mm., and the coefficient of variation was 3.69. In another Newtown series of 36, measured at 14 days, the length varied from 0.720 mm. to 1.134 mm., the average was 0.948 mm., and the coefficient of variation was 12.26. It is probable that the larger eggs produce relatively larger embryos. Künkel (1916) has proved this to be the case in *Arion* and in other land pulmonates. But it does not *necessarily* follow that the individuals which are larger at the time of hatching remain comparatively larger throughout their later lives.

The data of size as indicated by length are given in Table VII for the successive ages of representative broods of *palustris;* the LP1 and LP15 families belonged to the Newtown group while LP63 was a Croton snail. The figures are self-explanatory. They show how the illustrative families differ at the several age-intervals, and how their averages tend to converge in the latter portions of the life cycle. As a matter of detail, in some instances the average size is sometimes less at an older age than it was at an earlier time owing to the death of relatively larger individuals; a case in point is LP15-A at 400 days. In general, absolute variation as indicated by its coefficient is relatively greatest at the 80-day stage and it diminishes as the animals grow older, although there are notable exceptions.

The figures of Table VII and the curves of Fig. 2 show that the contrasted Newtown and Croton groups differ in their modes of growth. The Newtown animals generally increase in size at a slower rate at the outset, but they become comparatively larger at later times and they attain their maximum dimensions at about 320 days. The Croton snails have a shorter life-span as a rule, and they reach their lesser limits of growth by about 240 days—a point that is brought out more definitely in the subsequent section on longevity. The few individuals in every

TABLE VII

Data of size and growth of representative families of the second generation

Age	Family	No.	Arithmetical mean	Standard deviation	Coefficient of variation
days			*mm.*		
80	LP1–B	38	7.657	1.072	14.00
	LP15–A	11	9.463	2.866	30.29
	LP15–S	23	7.000	1.206	17.23
	LP63–K	74	8.605	2.257	26.23
160	LP1–B	35	10.011	1.463	14.62
	LP15–A	9	13.922	1.127	8.09
	LP15–S	22	21.368	1.317	6.16
	LP63–K	47	17.772	2.071	11.66
240	LP1–B	9	16.622	2.960	17.81
	LP15–A	5	21.000	3.130	14.90
	LP15–S	12	24.525	1.879	7.66
	LP63–K	20	18.525	1.394	7.52
320	LP1–B	5	21.760	2.365	10.87
	LP15–A	3	22.867	3.412	14.92
	LP15–S	7	24.415	1.397	5.72
	LP63–K	4	16.650	0.870	5.22
400	LP1–B	5	22.700	1.227	5.40
	LP15–A	2	21.400	2.545	11.90
	LP15–S	1	24.000	.....	.....
	LP63–K	0	.....	.....	.....

family that live beyond the times specified may not increase in size, but if they do, the increments of growth are small.

The contrasted distinctions of the Newtown and Croton groups are consistently the same in all of the material of the second generation. The series described in Table VIII differ somewhat from those of Table VII. The Croton group as a whole is definitely larger in size at 80 days while the members of the Newtown group are the larger at 160 days and 240 days with equal definiteness. An additional calculation

TABLE VIII

Comparative Sizes of the Newtown and Croton snails of the second generation

	N	$\bar{X}$	*F*	Significance
		mm.		
Size at 80 days				
Newtown	38	7.66		
Croton	74	8.60		
			5.981	positive
Size at 160 days				
Newtown	58	19.97		
Croton	47	17.77		
			11.570	positive
Size at 240 days				
Newtown	32	21.37		
Croton	23	18.41		
			13.865	positive

of 80-day sizes was made for the entire populations belonging to this generation with corroborative results; specifically, the mean size for the Newtown group was 7.150 mm. and the mean of the Croton group was 8.845, with a value for *F* of 15.662 that is certainly positive in significance.

When the third and fourth generations were analyzed (Table IX and Table X), the Newtown and Croton groups showed the same distinctions so far as the conditions at 80 days are concerned. At the 160-day period, the Croton snails were again smaller collectively in the third generation, but they were relatively larger in the fourth generation, although in neither case were the differences significant. In two respects the relations at 240 days were not the same as they were in the second

TABLE IX

Comparative sizes of the Newton and Croton snails of the third generation

	N	$\bar{X}$	*F*	Significance
		mm.		
Size at 80 days				
Newtown	261	6.67		
Croton	19	9.94		
			20.461	positive
Size at 160 days				
Newtown	251	16.73		
Croton	18	15.91		
			1.284	none
Size at 240 days				
Newtown	222	18.07		
Croton	9	16.58		
			3.691	none

TABLE X

Comparative sizes of the Newtown and Croton snails of the fourth generation

	N	$\bar{X}$	F	Significance
		mm.		
Size at 80 days				
Newtown	132	5.65		
Croton	86	7.69		
			36.645	positive
Size at 160 days				
Newtown	44	14.69		
Croton	43	14.98		
			0.210	none
Size at 240 days				
Newtown	38	17.13		
Croton	9	19.27		
			9.139	positive

generation. In the first place, the Croton snails did not differ significantly from the Newtown group although they were collectively smaller as before. In the second place, the Croton snails proved to be definitely *larger* at 240 days than the Newtown animals of the same age.

There is no question that the Newtown and Croton groups differ distinctly in mode of growth and in relative size during the earlier months of their life cycles, in all of the pedigreed generations. The circumstances during the later months of life are not so consistent. The reasons why the later generations fail to agree with the second generation are unknown. It may be that unconscious and unavoidable selection is responsible in part, and it may also be that the conditions of laboratory culture exert a modifying influence on the rates of growth in the course of time. However, the Newtown and Croton groups do show differences in certain physiological qualities of growth during their earlier lives, and these differences indicate that the two groups are justly to be regarded as distinguishable geographical races.

TABLE XI

Correlations of size and growth in Newtown snails of the third generation

Characters	N	r	F	Significance
Size at 80 days and size at 320 days	87	+.582	43.535	positive
Size at 80 days and increment of size from 80 to 160 days	87	−.719	91.075	positive
Size at 320 days and increment of size from 80 to 160 days	87	+.216	4.160	positive

Certain correlations of size and mode of growth were determined for some of the series, and the results are given in Table XI. The figures relate to random samples of the Newtown group of the third generation. Size at 80 days and size at 320 days are positively and definitely correlated. In general, the individuals which grew more slowly from the time of hatching to the age of 80 days were the smaller at the last. In other words, the animals tend to retain their respective positions in the graduated scale, from an early age to the virtual end of growth.

The increment of growth from 80 days to 160 days is *negatively* and significantly correlated with size at 80 days. It is clear that the larger snails of the 80-day array grew more slowly during the following interval in question in comparison with the smaller ones, even though the latter generally failed to overtake the former, as the previous correlation has shown. Obviously the form of the curve of growth varies. In some animals it rises rapidly and more precociously to the 80-day stage and thereafter the rate of increase gradually diminishes. In other instances the curve rises more slowly at first, and the transition to a period of more rapid growth falls beyond the 80-day stage. The positive correlation between size at 320 days and the growth increment from 80 days to 160 days is an incidental feature of some interest.

The average age at the onset of fertility is definitely correlated with size at 80 days, with a negative sign. The snails which were relatively larger at 80 days were generally the first to produce eggs. In a Newtown random sample of 12, r was —.766 and F was the significant figure of 14.238; in a comparable random sample of 63 Croton snails r was — .737 and the value of F, namely, 68.242, was also a significant figure. The two groups show exactly the same correlations even though they differ markedly in their mean ages at the onset of fertility as shown in the previous section.

Longevity

Discussion of the interesting problem of longevity is here confined to the contrast between the Newtown and Croton groups of *palustris*. No data are available for the members of the parent generation because their exact ages when they were brought into the laboratory were unknown; but the following generations have provided abundant information on the length of life of the snails from the date when the eggs were

laid to the time of death. No records were obtained for the animals that died before the age of 80 days, the time of isolation; therefore our calculations are based on random samples of those snails which were alive at the age specified.

From the figures of Table XII it is obvious that the Newtown snails of the 80-day population lived longer than the comparable series of Croton snails. The differences between the two groups are consistent and significant throughout all three generations, and they provide additional evidence that the Newtown and Croton groups constitute different geographical races. A minor point of interest is that erosion of

TABLE XII

Comparative length of life of the Newtown and Croton snails

Generation	N	$\bar{X}$	F	Significance
		days		
Second				
Newtown	69	245.13		
Croton	71	194.87		
			12.155	positive
Third				
Newtown	23	330.82		
Croton	22	216.77		
			39.567	positive
Fourth				
Newtown	31	332.39		
Croton	23	206.83		
			25.962	positive

the shell—an indication of senescence—often occurred in the Croton snails at or before 240 days, while in the Newtown snails it was seldom observed until after 320 days.

A positive and significant correlation between size at 80 days and age at death is revealed by the records. In a random sample of Newtown snails numbering 53, r was $+ .271$ and the value F was 4.200. In general, the snails which grew more rapidly before the 80-day interval lived longer than those with a slower rate of growth in the initial stages. There is no definite correlation between size at 320 days and age of death; in a random sample of 53 individuals, the r was $+ .185$ and F was 2.367, which is too small to be significant.

Sterility

The occurrence in *palustris* of numerous individuals that did not reproduce raises questions of considerable interest. The treatment of the problems of their sterility encounters certain difficulties, partly because the age when the contrasted fertile animals begin to produce eggs is widely variable.

In the case of the original or parent generation, 19 out of 34 Newtown snails (57.3 per cent) died without laying any eggs although they grew to full size, while 31 of the 45 Croton snails (68.9 per cent) were also sterile. Although their proportionate numbers varied from family to family, some of the members of the later generations failed to produce eggs although they lived well beyond the *average* age of onset of fertility of their respective groups. In the following discussion, however, only those individuals which lived beyond the *last* onset among their associates in the group, without laying eggs, are treated as sterile.

When the fertile and the sterile series were compared on the basis of relative size at successive age intervals (Table XIII and Table XIV), the members of the productive group were uniformly the larger at all periods of the life cycle. The Newtown and Croton series displayed no differences in these relations. But the two geographical series are unlike as regards longevity (Table XV). The general age at death of the fertile animals was definitely greater in the case of the Newtown animals; in the case of the Croton series, however, the difference between the fertile and sterile components was not statistically significant, although here again the animals which produced eggs were the larger collectively. Hence sterility is certainly associated with smaller size and shorter life in the *palustris* from the Newtown area, while it is associated with smaller size, but not with shorter life, in the Croton group.

Our animals were isolated at the age of 80 days before they reproduced and any eggs which were subsequently produced were certainly the products of self-fertilization. It is possible that some of the sterile individuals might have been fertile if they had been given an opportunity to mate, but there is no way to determine whether this might have been the case.

Self-fertilization in itself does not seem to have a detrimental effect upon the viability of the young or upon the productivity of fertile individuals in *palustris;* in fact, members of the fifth, sixth and seventh generations were even more prolific than those of the second generation. Crabb (1927) states that individuals of *Lymnaea stagnalis appressa* "reared in strict isolation reproduce as abundantly as do those in mass cultures." In the work of Colton and Pennypacker (1934) on *columella,*

TABLE XIII

Comparison of size of the fertile and sterile Newtown snails of the second generation

	N	$\bar{X}$	F	Significance
		mm.		
Size at 80 days				
fertile	56	6.90		
sterile	47	5.19		
			13.839	positive
Size at 160 days				
fertile	56	17.73		
sterile	37	16.10		
			8.640	positive
Size at 240 days				
fertile	173	18.53		
sterile	28	15.21		
			17.554	positive

93 consecutive generations were reared between 1911 and 1934 with self-fertilization throughout this period of years, and at the end the animals were hardy and prolific. Incidentally, sterile individuals are far more infrequent in *columella* than they are in *palustris*. Boycott, Diver, Garstang and Turner (1930) found that in *Lymnaea peregra* the animals would mate and cross-fertilize if they were given an opportunity to do so, but if they were reared in isolation they would produce fertile eggs equally well although at somewhat older ages.

But the point of greatest interest is that in our material of *palustris* absolute sterility is associated with smaller size. This suggests that the

TABLE XIV

Comparison of size of fertile and sterile Croton snails

	N	$\bar{X}$	F	Significance
		mm.		
Size at 80 days				
fertile	63	10.09		
sterile	28	7.73		
			38.333	positive
Size at 160 days				
fertile	51	17.57		
sterile	10	14.51		
			9.045	positive
Size at 240 days				
fertile	40	18.09		
sterile	13	15.67		
			22.559	positive

failure to lay eggs is due to retarded development or to an actual constitutional incapacity and not to the lack of an opportunity to mate. It is possible that real recessive defects involving the reproductive apparatus and its functions may exist, and that inbreeding might therefore increase the relative numbers of infertile individuals.

Summary

Variation in *Lymnaea palustris* is exhibited in fertility, rate of growth, size, and longevity. Individual, family and group differences recur in successive generations in spite of the essential uniformity of the laboratory conditions of culture; hence their genetic causation seems probable.

Sample populations collected from two different localities and habitats were analyzed by the F test for significant differences in fertility, growth, and longevity. The results demonstrated the reality of definite differences in all three categories.

The study of group differences was continued throughout three consecutive offspring generations, and in each of these the differences demonstrated in the parent generation were found to be repeated. The two local populations are therefore to be regarded as different geographical races.

Significant and positive correlations were found between the total number of eggs laid and the age at death, between the number of eggs laid and the length of the fertile period, and between the length of the fertile period and age at death.

Extreme types of slow-growing and fast-growing individuals were found in every group with intermediates of great variety. When arranged in order of size at 80 days, the snails exhibited a tendency to

Table XV

Comparison of age at death of the fertile and sterile snails of the second generation

	N	$\bar{X}$	F	Significance
		days		
Newtown group				
fertile	157	272.94		
sterile	34	223.29		
			9.991	positive
Croton group				
fertile	62	209.37		
sterile	24	196.04		
			1.053	none

keep their respective places throughout the rest of the life cycle, as indicated by the positive correlation between size at 80 days and size at 320 days. The rate of growth between 80 days and 160 days is inversely correlated with size at 80 days. The critical period in the growth curve occurs relatively early in the life cycle when the individual mode of growth appears to be established.

The onset of fertility is negatively correlated as to time with size at 80 days. The correlation in question is the same in the two local series, despite their demonstrated differences in size at 80 days and in age at the onset of fertility.

Some of the individuals in each population were sterile. When a study was made to ascertain whether such animals differed significantly from the fertile snails, the F test showed that they were collectively smaller than the productive individuals and that their lives were shorter.

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SOME OBSERVATIONS REGARDING THE NUCLEOLUS AND CYTOPLASM IN LIVING MARINE EGGS

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An attempt to determine further the chemical nature of nucleoli, which arose in connection with a survey of the nucleolus, now in press, has led to the following observations. Marine eggs were chosen as most suitable for the purpose. In fresh eggs of *Asterias forbesii* mounted in sea water under an immersion lens the nucleolus is relatively large and generally appears to contain two immiscible substances, the central droplet appearing faintly yellowish and more highly refractive than the surrounding colorless part. The central part, or nucleolinus, is sometimes in several droplets. The peripheral part is generally uniform, but in some nucleoli has a granular, in others a reticulate appearance. In some nuclei these two parts become separated as two unequal spheres, the nucleolinus being the smaller in volume.

In eggs of *Mactra solidissima* the nucleolus similarly consists of two unequal parts, but the nucleolinus instead of being concentrically placed is generally eccentric, touching one side of the nucleolar surface from the inside. It may contain one or more distinguishable globules or vacuoles. Not infrequently there are two nucleoli of unequal size, the larger containing as usual a more or less spherical eccentrically placed nucleolinus.

It is now known that the nucleolus or plasmosome normally contains no chromatin, but the two substances here observed evidently correspond to the basiphilic and oxyphilic substances so frequently described in nucleoli by animal histologists and by cytologists in animal eggs (see Wilson's "Cell," third edition, p. 270).

Attempts to obtain tests for lipoids from the nucleolus gave negative results. When fresh *Asterias* eggs are treated with Sudan III (0.1 per cent in 95 per cent alcohol) the egg cytoplasm stains yellow and the reagent is precipitated in orange-colored masses in the sea water around the eggs. While this confirms the presence of lipoids in the cytoplasm, the nucleus remains clear and there is no certainty that the color of the faintly yellowish nucleolinus is increased. Eggs which had

been killed by heating for five minutes on a water bath at 60° C. give the same result. When fresh *Mactra* eggs were treated with Sudan III the cytoplasm became pale yellow and small yellow aggregates formed just under the egg membrane, but no effect could be detected in the nucleolus. Similarly, when eggs of *Mactra* and *Asterias* were treated with 2 per cent osmic acid, no blackening effect on the nucleolus could be certainly observed, although the cytoplasm of *Mactra* became muddy and that of *Asterias* became yellowish with a delicate network of black. When fresh eggs of *Mactra* are dried on the slide by flaming and then treated with Scharlach R in alcohol the cytoplasm stains pink, showing the presence of fats, but the nuclei and nucleoli remain clear. When fresh *Mactra* eggs are mounted in chloroform on a slide, they become so granular that the nucleolus cannot be observed. If eggs are flamed and dried, then chloroform added, the nucleolus remains unchanged, although this reagent would be expected to dissolve any fats.

These results seem to negative the view that the nucleolus, at any rate of animal eggs, commonly contains ordinary lipoids.

Ruthenium red is regarded as a characteristic reagent for volutin in animals and pectin in plants. When a 0.2 per cent aqueous solution was added to fresh *Mactra* eggs the cytoplasm became more or less pink, which may have been merely an *intra vitam* stain, but the nucleolus remained uncolored.

Claude (1940) has found that by centrifuging normal and tumor cells, particles are separated—probably mitochondria—which contain phospholipids.

To test the possible presence of phospholipids in the nucleoli of animal eggs, about 15 cc. of Feulgen solution was added to four drops of *Asterias* eggs in sea water. Without any hydrolysis, the egg cytoplasm begins to be colored in less than a minute, the characteristic magenta color gradually getting deeper while the nucleus remains uncolored and the nucleolus in many eggs becomes invisible, probably dissolving. On standing, the egg cytoplasm becomes brilliant magenta, the nuclei remaining clear. When a mass of the polygonal ovarian eggs is similarly treated with Feulgen, the color appears at once and is even more intense, indicating a greater concentration of some substance containing a free aldehyde group. The peripheral free eggs in such a mass are at first uncolored. Then the egg membrane appears magenta. When 5–6 cc. of Feulgen were added to three drops of concentrated eggs in distilled water, the nucleolus was dissolved at once and the cytoplasm shortly began to take on the characteristic color. About half the eggs were cytolyzed by the fresh water.

The appearance of the magenta color in unhydrolyzed cells indicates the presence in the cytoplasm of substances having a free aldehyde group. Such a class of lipoidal substances has been found by Feulgen and Voit (1924) in the cytoplasm of animal tissues and of an intestinal ciliate. The substance plasmalogen is insoluble in water, but with acids or sublimate splits off an aldehyde, plasmal, which gives the violet coloration with Schiff's reagent. The rate of coloration in any case will then depend on the rate of change from plasmalogen to plasmal, which may depend in turn on the fineness of colloidal dispersion of the plasmalogen in the cell.

Plasmal was later found (Feulgen and Bersin, 1939) to be a mixture of higher aldehydes of the known fatty acids, was extracted from horse flesh with alcohol, and a formula for plasmalogenic acid was obtained. This nitrogen-free acid resembles the phosphatid acids obtained from cabbage and spinach by Chibnall and Channon (1927). It appears that the substances in the cytoplasm of the starfish and other marine eggs, which give this strong magenta coloration with Feulgen, without hydrolsis, are aldehydes belonging to the class of acetalphosphatids. The classical brain phosphatids were found to contain as much as 20 per cent of acetalphosphatid. Whether they are contained in "mitochondria" cannot be stated. However, observations of *Chaetopterus* eggs treated with Feulgen under a 2 mm. immersion lens show that many of the granules in the cytoplasm take the magenta color. In crushed eggs some granules are deep magenta, some pale, some uncolored. There is also a diffuse pink color in the cytoplasm. Some of the magenta granules are of maximum size, some very minute, and the smallest visible granules take the color most intensely while some granules of all sizes remain uncolored.

Although the cytoplasm of the *Chaetopterus* egg is colorless, in Feulgen these eggs take the color much more slowly than Asterias eggs and only show it definitely after nearly an hour. The area of the maturation spindle remains clear. By the next day the cytoplasm is intensely purple. The muscle fibres in the fans of *Chaetopterus* are colored in the same way, the surrounding tissue remaining unaffected. *Mactra* eggs in Feulgen begin to show the color in about ten minutes and the cytoplasm is definitely colored in about forty-five minutes. *Arbacia* eggs also develop the magenta color slowly, perhaps because it is obscured by the red pigment already present in the cytoplasm, but it finally appears in its full intensity.

The plasmalogens were found by Feulgen and Voit to be insoluble in water, soluble in alcohol and organic solvents. To test these proper-

ties, eggs of *Arbacia* and of *Chaetopterus* were killed by being heated in a water bath, then washed in sea-water, fifteen changes being made in about fifty-four hours. If Feulgen is then added to these *Arbacia* eggs, they shortly begin to change to orange color; in half an hour many of the eggs are magenta and by next morning all show this color. In *Chaetopterus* eggs, after washing, the cytoplasm is granular but none seem cytolyzed. When Feulgen is added, the cytoplasm of some eggs begins to show a faint rosy tint after five minutes and most of them are full magenta in half an hour, but the color is less intense than in unwashed eggs. A few eggs remained quite colorless. While these plasmalogens are then relatively insoluble in water, there is evidence that a small amount has been washed out, but the sea water from washed eggs, when tested with Feulgen, gave negative results.

Fresh *Mactra* eggs were left two days in 95 per cent alcohol. Ordinary alcohol quickly turns pink when Feulgen is added to it, because of the aldehyde present; and it was not evident that the alcohol from these eggs changed color more quickly or more deeply. But the eggs, when tested with Feulgen, were only faint pink after five minutes; after an hour the cytoplasm was pale pink and the nucleus had changed its appearance, being pale pink, the nucleolus uncolored. From this it appears that most of the plasmalogens have been extracted from the cytoplasm by alcohol. *Mactra* eggs were flamed on the slide, then placed in 1 per cent sublimate for three minutes, then washed in distilled water and mounted in Feulgen. In a few minutes the cytoplasm is pink and after an hour the eggs remain rather pale pink. If, as stated by Feulgen and Voit, sublimate accelerates the separation of the aldehyde plasmal from plasmalogen, a more intense coloration would be expected.

From these preliminary experiments it can be stated that the aldehyde substances in the cytoplasm of invertebrate animal eggs agree in many respects with those lipoidal substances found to be of widespread occurrence in animal tissues, but they may differ in certain particulars. The absence of phospholipids or other lipoids from the nucleoli seems to be definitely shown. Attempts with *Mactra* eggs to throw out the nucleolus, in order to make mass tests, were unsuccessful at a centrifuge speed of about 70,000 g.

It has already been mentioned that in eggs of *Asterias* and *Mactra* the nucleolus consists of two unequal parts, one enclosed within the other. These are soluble in tap water; but if water is added slowly while the eggs are under observation with an immersion lens, it can be seen that they show marked differential solubility, the internal "nucleolinus" generally remaining long after the outer part has disappeared.

During this process the two parts may become separated. Gersch (1940) has found that in frog's eggs when the nuclei are separated out the nucleoli are soluble in distilled water.

Egg cells and oogonia of *Fucus* in sea water are negative to Feulgen at ordinary temperatures, showing no change in color after an hour and more. When exposed to air, after Feulgen treatment the thallus turns purple. At a higher temperature both egg cells and the colorless strands in the medulla of the thallus become pink or magenta and dark magenta bodies appear in the cytoplasm of these medullary filaments. It has not been possible to make any further experiments on this subject.

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THE OSMOTIC PROPERTIES OF THE NUCLEUS

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INTRODUCTION

In spite of the fact that extensive investigations have been carried out upon the osmotic behavior of cells and tissues, comparatively little attention has been devoted to the study of the osmotic properties of the nucleus. There is, however, considerable evidence in the literature that the nuclear membrane is semi-permeable. Thus Shinke (1937) was able to demonstrate osmotic swelling and shrinking for the nuclei of perhaps twenty different species of plant cells. He found too that the nuclei of the intestinal epithelium of *Triturus pyrrhogaster*, the nuclei of the salivary gland cells of *Chironomus dorsalis* and the nuclei of the first spermatocytes of *Tryxalis nasuta* react osmotically. Reversible swelling and shrinking of the nuclei of the salivary gland cells of dipterous larvae have been observed by Buck and Boche (1938). Olivo (1938) reported that the nuclei of myocardial cells swell in distilled water. Küster (1932) produced "Niederschlagsmembranen" upon the surface of nuclei of various plant cells by treatment with injurious agents, and states that these membranes are semi-permeable. Further references to the osmotic properties of the nucleus under pathological conditions will be found in Küster's paper. Finally, Beck and Shapiro (1936), studying the permeability to water of the starfish egg and its germinal vesicle, made certain conjectures concerning the relative amounts of osmotically inactive substance in cell and nucleus.

The cursory observations reported above are supplemented by two papers of a more rigorous, quantitative nature. One of these consists of the extensive series of investigations conducted many years ago by Hamburger (1904). He not only made semi-quantitative determinations of the degree of swelling and shrinking of the nuclei of different types of gut epithelia in anisotonic NaCl solutions, but even succeeded in estimating the amount of osmotically inert material ("Gerüstsubstanz," Hamburger; "colloid substance," Hardy) in the frog spermatozoön. In addition he noted that isolated nuclei can react osmotically.

Probably the most extensive data compiled on the osmotic behavior of the nucleus are those of Skowron and Skowron (1926) for the germinal vesicle of the egg of *Sphaerechinus granularis*. In this work the diameters of the cell and germinal vesicle were measured in different dextrose solutions whose freezing-point depressions were known. They found that the cell and nucleus are impermeable to dextrose and that both swell in hypotonic solutions. They state further that, although the cell as a whole shrinks in a hypertonic dextrose solution ($\Delta T = -2.57°$ C.), the nucleus undergoes no diminution in volume. This latter observation can only be interpreted as meaning that the germinal vesicle of the *Sphaerechinus* egg does not behave osmotically; a conclusion at variance with the observations to be presented here on the osmotic properties of the germinal vesicle of the immature egg of *Arbacia punctulata* and that of the mature, unfertilized egg of *Nereis limbata*.

Materials and Methods

The immature eggs of *Arbacia punctulata* and the mature, unfertilized eggs of *Nereis limbata*—two marine invertebrates—were used in these experiments. These were chosen for study because the nucleus (germinal vesicle) of each is large and spherical, and its volume can easily be determined.

Immature *Arbacia* eggs were obtained as follows. The ovaries were removed from the test to a dish of sea water, whereupon the mature eggs were extruded. A fine pipette was then inserted into the "collapsed" ovary and many immature eggs, of various sizes and in different stages of development, were removed. Since these eggs are aspherical in the ovary and only slowly round up in sea water, it was found advisable to allow them to remain in sea water for about ten minutes before starting an experiment.

The immature eggs were transferred to a glass chamber of approximately 25 cc. capacity and a suitable cell was chosen for microscopical study. Measurements of the diameters of the cell and its germinal vesicle were made using one of two methods: projection by means of a camera lucida, or photographically. The photographic method will be described in detail in a future paper dealing with the permeability of the nucleus to water. Measurements were consistent to 0.4 μ with either technique. No attempt was made to maintain a constant temperature.

After determining the diameters of the cell and its nucleus in 100 per cent sea water, the superfluous sea water was removed with a micropipette until less than 0.5 cc. remained directly under the water

immersion lens (40 ×). The experimental solution was now carefully added and the equilibrium diameters of the cell and its nucleus determined 30 minutes later. Then the experimental solution was removed, sea water added, and the measurements once more carried out upon the attainment of equilibrium.

In general the same procedure was followed in the experiments with the *Nereis* eggs. These were obtained by snipping the body of the worm in a finger bowl containing sea water, and were then arranged for microscopical study as described for the *Arbacia* material. In this case, however, only the nuclei were measured. Moreover, instead of carrying out a complete experiment, involving reversible swelling and shrinking of a single nucleus, the nuclei of 50 or more cells were measured in any one experiment. In order to reduce the biological variations, only the eggs from one animal were used for an experiment.

The solutions were prepared by appropriately diluting sea water with distilled water (hypotonic solutions), and by concentrating sea water by forced evaporation to obtain hypertonic solutions whose freezing-point depressions had been previously determined (Churney, 1940).

In addition to the experimental techniques already described, another series of experiments was carried out on the *Nereis* nuclei. These were designed to determine whether or not the final volumes reached by the nuclei at equilibrium in anisotonic solutions are dependent upon the path followed. The experiment was very simple. Eggs from a single female were divided into two sets and about 50 nuclei measured in each. One group was then transferred to 80 per cent sea water; the other to 60 per cent sea water. A half-hour later 50 nuclei in the 80 per cent sea water solution were measured. The 80 per cent sea water was then removed from the chamber and a 60 per cent solution substituted. Following attainment of equilibrium 30 minutes later, the diameters of 50 nuclei were measured in each of the dishes containing 60 per cent sea water. The same experimental procedure was performed using 70 and 50 per cent sea water.

Results and Discussion

In accordance with the treatment of the subject by Lucké and McCutcheon (1932), two conditions must be satisfied for a cell or nucleus to behave as an osmometer. First it must have a semi-permeable membrane. This may be defined as one which permits the passage of the solvent, water, but excludes the solutes, mainly salts. To test the assumption of a semi-permeable membrane, data have been compiled showing the volumes attained by cells and nuclei

upon return to sea water after previous immersion in hypertonic or hypotonic solutions. If the solutes do not penetrate, the initial volume of the cell or nucleus, V_0, in sea water will be equal to the equilibrium volume, V_0', assumed upon return to sea water after immersion in an anisotonic solution. Discrepancies serve as a measure of the deviation of the membrane from semi-permeability. In case of equal and reversible exchange of solutes during swelling and shrinking this relationship would be camouflaged.

In the second place the osmotic behavior of the cell must follow the Boyle-van't Hoff law. This principle states that at equilibrium the product of the cell volume by the osmotic pressure of the external solution is a constant for all concentrations; or,

$$PV = K.$$

At equilibrium the osmotic pressure within the cell is considered equal to that of the external solution. Since the cells are always in equilibrium with 100 per cent sea water, P_0V_0, the equation takes the form

$$P_0V_0 = P_1V_1,$$

where P_1V_1 represents the pressure-volume product of the cells at equilibrium in anisotonic solutions.

As pointed out many years ago by Hamburger (1904), and extensively investigated by Lucké and McCutcheon (1932), the Boyle-van't Hoff law holds only for ideal solutions. This means that, for the cell and nucleus to obey this rule perfectly, the cell contents would have to be infinitely dilute and contain no osmotically inactive material. To correct for the "non-solvent volume," Lucké and McCutcheon introduced a factor "b," *which is independent of the external osmotic pressure.* The equation then becomes

$$P_0(V_0 - b) = P_1(V_1 - b).$$

Data for the immature *Arbacia* egg are presented in Table I. Agreement with the Boyle-van't Hoff law is determined by equating the values for P_0V_0 with those for P_1V_1 (columns 3 and 4 respectively) for any one relative pressure (column 2). The deviations are given in column 5. The results indicate: (1) that there is good adherence to the Boyle-van't Hoff law in the case of solutions whose osmotic pressure is close to that of sea water (1.13, 0.9, 0.8 times); and (2) that as one approaches greater dilutions, the deviations increase considerably and show a decided drift. The negative signs in column 5 signify that, in the case of the hypotonic solutions, the cells do not swell to the required volumes. It should be pointed out that there is some evidence that 50 per cent sea water is injurious. In the other

TABLE I

Immature eggs of Arbacia punctulata

Number of Cells	Relative Pressures	P_0V_0	P_1V_1	Percentage of Deviation
13	1.13	189.9	194.9	2.6
21	0.9	191.0	185.9	−2.7
26	0.8	198.8	190.8	−4.0
33	0.7	217.7	198.6	−8.8
40	0.6	208.8	184.8	−11.5
34	0.5	214.0	178.2	−16.7

direction it was found that a hypertonic solution equivalent to 131.0 per cent sea water represents the limiting concentration, because of the fact that in higher concentrations the cells form blebs and are no longer spherical.

In Table II is presented a similar set of data for the egg of *Sphaerechinus granularis* taken from the data of Skowron and Skowron (1926). Columns 1, 2, 3, and 6 are arranged from their various tables; columns 4 and 5 have been compiled by the writer. The freezing-point depression of the sea water at Naples is given as − 2.28° C., so that "$\Delta T \cdot V_1$" = 74.51 (column 4) is really P_0V_0. The remaining figures in this column are P_1V_1.[1] The agreement between the values for P_1V_1 and that for P_0V_0 (= 74.51) is fair except in the case of the solution whose freezing-point depression is − 1.26° C., which is equivalent approximately to 55 per cent sea water. In the case of the most hypotonic solution (ΔT = − 0.97° C.), injury is manifested by loss of semi-permeability and by cytolysis. That the data in general are in harmony with the Boyle-van't Hoff law can be seen from their "Diagramme 1" (not reproduced), in which "Masse" is plotted as the

[1] The arbitrary figures ("cm.³") for the volumes given by Skowron and Skowron may readily be converted into cubic micra by noting that, "Nous avons tracé les contours des oeufs à l'aide d'un appareil à dessiner à un grossisement de 390 fois; à la hauteur de la platine du microscope." Thus for a cell whose volume is 32.683 cm.³, we have

$$V = \frac{32.683 \times 10^{12}}{(390)^3} = \frac{32.683 \times 10^{12}}{5.9319 \times 10^7} = 550 \times 10^3 \mu^3$$

The diameter of such a cell will be given by,

$$D = \sqrt[3]{\frac{6V}{\pi}} = 101.6\mu.$$

Likewise the volume of the nucleus of this cell is given as 4.268 cm.³ (Table IV); its volume in cubic micra is

$$V = \frac{42.68 \times 10^{11}}{5.9319 \times 10^7} = 70.3 \times 10^3 \mu^3,$$

and its diameter, 51.2μ.

TABLE II

Eggs of Sphaerechinus granularis
(after Skowron and Skowron, 1926)

Number of Cells	Pressures ΔT	Equilibrium Volumes "cm.³"	$\Delta T \cdot V_1$	Percentage of Deviation	Remarks
14	2.28	32.683	74.51		
7	1.73	41.293	71.43	−4.1	
11	1.70	42.673	72.54	−2.6	
12	1.26	50.871	64.10	−14.0	
10	1.08	67.448	72.85	−2.2	cytolysis
10	0.97	103.735	100.63	35.0	cytolysis

ordinate and "Δ" as the abscissa. The resulting curve is a fair rectangular hyperbola, as is required if the equation $P \cdot V = K$ is satisfied. In addition they report that the cell as a whole shrinks in hypertonic solutions.

A discussion of the "non-solvent volume," b, will be reserved until after the data for the nucleus have been presented.

In Table III are arranged the data for the osmotic properties of the germinal vesicle of the immature *Arbacia* egg. A test of the semi-permeability of the nuclear membrane may be made by comparing the values of P_0V_0 with those for $P_0'V_0'$ (columns 7 and 8 respectively) for any one relative pressure (column 2). The deviations in percentage are given in column 9. It is a matter of regret that more nuclei were not measured, yet it appears certain that the nuclear membrane is truly semi-permeable.

Comparing the deviations of the values for P_0V_0 with those for P_1V_1 (column 5) one arrives at the following conclusions: (1) adherence to the Boyle-van't Hoff law is good; (2) as in the case of the figures

TABLE III

Germinal vesicles of Arbacia punctulata

Number of Nuclei	Relative Pressures	P_0V_0	P_1V_1	Percentage of Deviation	Number of Nuclei	P_0V_0	$P_0'V_0'$	Percentage of Deviation
6	1.13	30.8	28.1	8.8	3	34.0	32.0	5.9
17	0.9	20.5	20.4	−0.5	10	20.5	20.4	−0.5
46	0.8	27.5	27.5	0.0	13	28.2	27.9	−1.1
51	0.7	27.5	26.8	−2.5	24	28.1	28.7	2.1
53	0.6	28.0	26.4	−5.7	19	27.5	27.5	0.0
48	0.5	28.8	25.4	−11.8	8	28.0	27.9	−0.4

for the cell as a whole, there is better agreement for solutions whose osmotic pressure is close to that of sea water; (3) there is a marked drift in the deviations with increasing dilutions; and (4) the percentage deviations are considerably smaller for the nucleus than for the ce

as a whole. One is strongly tempted to draw the conclusion that the nucleus is a better osmometer than the cell.

For comparison with the results cited for the *Arbacia* nucleus, Table IV, which deals with experiments on the osmotic properties of

TABLE IV

Nuclei of Sphaerechinus granularis
(after Skowron and Skowron, 1926)

Number of Nuclei	Pressures ΔT	Equilibrium Volumes "cm.^{3}"	$\Delta T \cdot V_1$	Percentage of Deviation
14	2.28	4.268	9.74	
7	1.73	6.254	10.81	10.9
11	1.70	6.954	11.82	21.2
12	1.26	7.553	9.51	−2.4
10	1.08	10.384	11.21	15.1
10	0.97	6.018	5.84	−40.1

the germinal vesicle of the *Sphaerechinus* egg, has been prepared from the data of Skowron and Skowron. Columns 1, 2, and 3 have been taken directly from their tables and columns 4 and 5 compiled by the writer. The average of the equilibrium volumes in sea water (P_0V_0) is 9.74. All the other figures in column 4 correspond to P_1V_1. In this case the figures of column 5 indicate considerable deviation from obedience to the Boyle-van't Hoff law and contrast sharply with the results just presented for the germinal vesicle of the *Arbacia* egg

TABLE V

Germinal vesicles of Nereis limbata

Relative Pressure	Number of Nuclei, V_0	Number of Nuclei, V_1	P_0V_0	P_1V_1	Percentage of Deviation	Number of Nuclei, V_0'	$P_0'V_0'$	Percentage of Deviation
0.9	56	64	98.37	96.34	−2.06	27	95.90	−2.51
0.9	73	61	103.33	101.83	−1.45	60	107.62	4.15
0.8	51	43	101.14	94.21	−6.85	38	104.33	3.15
0.7	53	56	100.67	84.32	−16.24			
0.7	57	58	98.53	87.86	−10.83	20	95.77	−2.80
0.6	27	49	127.47	102.24	−19.78	51	123.82	−2.86
0.5	49	44	108.99	77.56	−28.83	35	116.63	7.01

(Table III). It may be, of course, that the germinal vesicle of the *Sphaerechinus* egg does not obey the Boyle-van't Hoff law. Indeed, their statement that the nucleus does not shrink in hypertonic solutions points definitely to this conclusion. However, it seems likely, as the investigators themselves admit, that accurate results cannot be obtained for the nucleus by their method. It would certainly be de-

sirable to have more information concerning the accuracy of their technique.

Evidence that the nucleus is an osmometer has been extended to the case of the germinal vesicle of the *Nereis* egg (Tables V and VI). The criterion of semi-permeability may be applied by comparing the values of P_0V_0 (initial volumes) with those for $P_0'V_0'$ (volumes assumed upon return to 100 per cent sea water after a swelling experiment). The data are presented in Table V, columns 4 and 8 respectively. All the deviations are greater than those for the *Arbacia* nuclei. The values fluctuate more or less at random about zero and indicate that the nuclear membrane is probably semi-permeable. The entry for 50 per cent sea water is difficult to understand, since its algebraic sign is the opposite of what one would expect if salts were being lost during the swelling process.

TABLE VI

Germinal vesicles of Nereis limbata

P_0V_0	P_1V_1 in 80% Sea Water	Percentage of Deviation	$P_1'V_1'$ in 60% Sea Water	Percentage of Deviation
A. 90.17	90.34	0.19	78.67	−12.75
B. 91.71			81.52	−11.11
P_0V_0	P_1V_1 in 70% Sea Water	Percentage of Deviation	$P_1'V_1'$ in 50% Sea Water	Percentage of Deviation
A. 96.44	81.95	−15.02	68.15	−29.33
B. 95.60			71.14	−25.58

In order to gain more information on this point, another sort of experimental attack was attempted. The procedure, already described under the heading "Materials and Methods," consisted in comparing the averaged volumes of two sets of nuclei brought by different paths into equilibrium with any given solution. In one case the nuclei were transferred directly from 100 per cent sea water to a diluted medium; in the other, only after a previous immersion in a solution of some intermediate concentration.

The reasoning underlying this experiment is based on the assumption that leakage is a function, among other things, of the concentration gradient. That is, the degree to which salts will tend to pass out of the nucleus will depend upon the rate of swelling. It may be expected, then, that the final volumes attained by the nuclei at equilibrium in any anisotonic solution will depend upon the path taken.

The results of these experiments show definitely, however, that the nuclei attain their final volumes quite independently of the path

traversed. Thus, for the series in 80 and 60 per cent sea water, the deviation from the volumes demanded by the Boyle-van't Hoff law is 11.11 per cent in the case of the nuclei transferred directly to 60 per cent sea water (Table VI, column 5). The value of the deviation for the nuclei that were placed in 60 per cent sea water only after being previously equilibrated with 80 per cent sea water is 12.75 per cent. The difference between the deviations is 1.64 per cent, which is equal to the difference between the averaged volumes of the nuclei in 100 per cent sea water (column 1).

In the case of the series in 70 and 50 per cent sea water, there is a difference between the deviations of 3.75 per cent (column 5) as a result of the two different methods of bringing the nuclei into 50 per cent sea water. The difference between the averaged volumes initially is only 0.84 per cent (column 1). Here the agreement is not as good as for the series in 80 and 60 per cent sea water, and it appears that the property of perfect semi-permeability may be lost at dilutions as great as 50 per cent sea water.

The degree to which the germinal vesicle obeys the Boyle-van't Hoff law may be determined from the data in Table V. Although biological variations of considerable magnitude occur (note, for example, the variations in the averages of the initial volumes, P_0V_0, column 4), the following conclusions are perfectly clear. First, there is better agreement with the Boyle-van't Hoff law at the lesser than at the greater dilutions; compare the values of P_0V_0 with those for P_1V_1 (columns 4 and 5 respectively) for any one relative pressure (column 1). Secondly, the magnitude of the deviations is greater for the nucleus of the *Nereis* egg than for that of the *Arbacia* egg. Thus the entries in Table V show deviations ranging from 10 to 15 per cent in 70 per cent sea water, whereas comparable deviations for *Arbacia* nuclei occur only at dilutions as great as 50 per cent sea water. The value of the deviation for *Nereis* nuclei in 50 per cent sea water is of the order of 25 per cent (Table V, column 6; Table VI, column 5). Finally, it may be mentioned that the nuclei shrink in hypertonic solutions, although data have not been compiled to test quantitatively this aspect of the problem. It may be concluded, then, that the germinal vesicle of the *Nereis* egg behaves as an osmotic system, though not to the same high degree as that of the *Arbacia* egg.

It is appropriate at this point to discuss the meaning of "b," the value of the osmotically inactive material, for these experiments.[2]

[2] Except where explicitly stated, the remainder of this article is concerned exclusively with the data for the *Arbacia* material. A complete set of statistical data for cells and nuclei is presented in Tables VII and VIII.

In a study of this sort it is desirable to have an exact idea of the variability of the material. The eggs chosen for experiment were restricted more or less to the

For one of the purposes of this work, in addition to the investigation of the osmotic properties of the nucleus, was to determine, if possible, the values of "b" for cells and nuclei at various stages during their growth. This would have given a physiological index of great importance for understanding intracellular growth. (A partial solution of this problem will be offered further on.) Unfortunately, the almost insurmountable difficulty of making great numbers of measurements on groups of cells of similar sizes was immediately encountered. Consequently it was necessary to abandon this particular phase of the investigation and to concentrate instead on determining the osmotic relationships of a rather heterogeneous population of cells and nuclei; the averaged volumes being used in the equations. Obviously, an "average" value of "b" for cells and nuclei of very different volumes has little or no physiological meaning.

Actually, the data for the nucleus indicate that there is an insignificant amount of osmotically inactive substance present. This is proved by testing statistically the agreement between the data and the Boyle-van't Hoff law. The analysis consists in determining the significance of the "difference between the means" (the values of P_0V_0 minus those for P_1V_1 for 80, 70, 60, and 50 per cent sea water). If the differences are not significant, then Boyle's law holds; and vice

later growth stages: the diameters of the cells vary from 65 to 80μ; those of their nuclei from 35 to 42μ (column 4, Tables VII and VIII respectively). From the coefficient of variation, C, (column 6) it can be seen that initially the variability, representing entirely variations in sampling technique, is of the order of $\pm$ 10 per cent. This figure is the same for cells and nuclei, indicating that variations in the nuclear volumes are associated with variations in egg volumes—a deduction to be expected on *a priori* grounds. In addition, the data show that the variations in the equilibrium volumes in anisotonic solutions are determined to a considerable extent by the variations in the initial volumes. From this the unexpected result emerges that ***the variation in the final volumes is independent of the concentration of the medium.***

Another set of statistics that is important is concerned with the correlation coefficient, $r_{V_0V_1}$. Indeed, the correlation between the initial and final volumes is in reality a measure of the degree of validity of the Boyle-van't Hoff law. For if the process followed this rule exactly, then theoretically a correlation coefficient, ρ, of + 1 would be expected for the supply. The effect of the great variation found in the samples will act, however, to reduce the value of $r_{V_0V_1}$ considerably. In other words, the correlation will tend always to *underestimate* the degree of association.

Some idea of the variations encountered in the correlations can be determined by use of the regression equations and standard error of estimate (Table IX). These equations are extremely useful since they indicate the most likely value, expressed in cubic micra, that the volume of a cell or its nucleus will attain when immersed in a hypotonic solution. The coefficient of V_0, the slope of the regression line, expresses, in cubic micra, the average change in V_1 accompanying unit change in V_0. Evidently the greater the correlation between V_0 and V_1 in the different solutions, the more nearly alike the values of these coefficients will be. The data show that not only are the values of the slope very much alike for different media, but that they are quite similar for cells and nuclei interchangeably. The variations around the regression line are given by the standard error of estimate which may be interpreted in the same way as a standard deviation.

versa. Because of the high correlation involved (Table VIII, column 7), it is necessary to make this test as sensitive as possible. To this end the standard error of the difference has been calculated from the following formula (Treloar, 1939, pp. 146–148):

$$\text{S.E.}_{\bar{d}} = \sqrt{\text{S.E.}^2_{P_0V_0} + \text{S.E.}^2_{P_1V_1} - 2r_{V_0V_1} \cdot \text{S.E.}_{P_0V_0} \cdot \text{S.E.}_{P_1V_1}}.$$

From the actual difference between the means and the standard error of the difference, the relative deviate has been determined and the probability read off from prepared tables.

When this test is applied to the data for the nucleus (Table VIII), it is found that the difference in the means of 0.02 for 80 per cent sea water can arise in 95 cases out of 100 merely through errors of random sampling. Such a difference, then, is insignificant and the simplified equation $P \cdot V = K$ suffices. On the other hand, this equation is no longer valid when the difference of 0.77 for 70 per cent sea water is considered, for here the 5 per cent level of significance is approached. In 60 and 50 per cent sea water the differences are highly significant. Nevertheless, the fact is inescapable that in 90 and 80 per cent sea water the value of "b" is zero,[3] and so the nucleus cannot be said to contain any significant amount of osmotically inert material.

The conclusion that the nucleus contains an inappreciable amount of osmotically inactive substance requires qualification. Certain pertinent viscosity data in the literature may be interpreted as indicating that a considerable amount of dispersed material exists in the nucleus. For the viscosity of a solution is related in a complicated fashion to the volume fraction of the solute. The viscosity of the germinal vesicle of the *Echinus* egg, a form closely allied to *Arbacia*, has been variously determined as being from two to ten times that of water (Heilbrunn, 1928; Harris, 1939). Employing any of the numerous equations relating viscosity to the volume fraction of the solute (the most satisfactory of which appears to be that of Kunitz, 1926: $\eta = \frac{1 + 0.5\,\varphi}{(1 - \varphi)^4}$) estimates of φ (b?) range from 10 to 40 per cent of the total volume. It should be noted that this treatment tacitly assumes that the term "osmotically inactive material," b, is synonymous with that of "volume fraction of solute," φ. This is, indeed, the conventional definition of "b" as employed by physiologists since its conception by Hamburger and Hardy. Actually, as measured by osmotic effects, the b-factor represents an *effective* rather than a real

[3] The same conclusions apply in the case of the germinal vesicle of the *Nereis* egg. This is shown by the agreement between the values of P_0V_0 and P_1V_1 in 90 per cent sea water (Table V) and in 80 per cent sea water (Table VI, columns 1 and 2). In the latter case the value of the deviation is only 0.19 per cent.

volume, and as such might well be some multiple of φ. A real discrepancy exists between the values of "b" and of "φ" or some multiple thereof.

There are in the nucleus only two formed structures which might be expected to contribute to the osmotic "dead space." These are the chromosomes and the nucleolus. By analogy with the behavior of other chromosomes, those of the germinal vesicle should behave osmotically.[4] The osmotic properties of the nucleolus will receive especial attention in a following section. In short, it hardly seems likely that any considerable proportion of "b" (or φ) can be due to the morphological constituents of the nucleus.

With respect to the cell as a whole, the situation is somewhat different. There can be no doubt that the differences between the means are real. Thus, for cells swollen in 80 per cent sea water, the chances are less than 1 : 10,000 that a difference of 7.92 could arise from sampling errors alone. One can calculate an "average" value of "b" and compensate for the deviations from the equation: $P_0V_0 = P_1V_1$. (Interestingly enough, the viscosity of the cytoplasm, as determined by centrifuge experiments, is probably very high—indicating a high value for φ.) The drift would still remain, although it would be smaller in magnitude. The largest deviation would again be found in the case of 50 per cent sea water. One could test for the significance of the difference between the means [values for $P_0(V_0 - b)$ minus those for $P_1(V_1 - b)$] as before. If the differences were insignificant, all the data would be accounted for by this simple modified equation and the analysis would be complete. If not, one would seek other causes to explain the discrepancies. It seems to me, however, that this sort of treatment is extremely deceptive in its implications, especially if no independent method for determining "b" exists.

In a previous section it was stated that a partial answer would be given to the question of the relation of the osmotic behavior of these immature ova and their germinal vesicles to their initial volumes. From the point of view of statistics the problem may be worded thus: Given the two variates, V_0 and V_1, where V_1 is a function of V_0, what relation does V_1 bear to V_0 as V_0 varies from low to high values?

Problems of this sort were investigated by Harris (1909). The solution is obtained from a consideration of the three possible cases for the values of r_{xz} as calculated by the formula

$$r_{xz} = \frac{r_{V_0V_1} - C_{V_0}/C_{V_1}}{\sqrt{(1 - r^2_{V_0V_1}) + (r_{V_0V_1} - C_{V_0}/C_{V_1})^2}}.$$

[4] The giant chromosomes of the salivary gland cells of *Sciara coprophila* and of *Chironomus* sp. swell and shrink reversibly in anisotonic solutions; unpublished data.

The three cases that arise are: (1) when $r_{zz} >$ zero, V_1 will tend to attain relatively larger values (i.e., obey the Boyle-van't Hoff law more closely) as V_0 increases from smaller to larger volumes; (2) when $r_{zz} <$ zero, V_1 will tend to attain relatively smaller values (i.e., deviate more and more from the Boyle-van't Hoff law) as V_0 increases in value; and, (3) when $r_{zz} =$ zero, it may be stated that small and large ova or small and large nuclei obey the Boyle-van't Hoff law to an equal degree.

The values of r_{zz} for all dilutions as calculated from the preceding formula are given in Tables VII and VIII, column 8. They are consistently negative in sign and of considerable magnitude, especially at the greater dilutions. The conclusion is drawn that smaller eggs and smaller nuclei (within the limited ranges of volumes investigated) tend to be better osmometers than larger eggs and larger nuclei.

TABLE VII

Immature eggs of Arbacia punctulata

Relative Pressures		Number of Cells	Averaged Volumes ±P.E.$_M$ μ^3	Ranges μ^3	Standard Deviation, σ; ±P.E. μ^3	Coefficient of Variation, C; ±P.E.$_C$	Correlation Coefficient, $r_{V_0V_1}$; ±P.E.$_{r_{V_0V_1}}$	r_{zz}
0.8	V_0	26	198.76±2.14	158.3–226.0	16.16±1.51	8.13±0.76	0.857±0.035	−0.100
	V_1		238.55±2.82	189.4–272.4	21.32±1.99	8.94±0.84		
0.7	V_0	33	217.73±3.03	170.0–261.6	25.79±2.14	11.84±1.00	0.848±0.033	−0.072
	V_1		283.65±4.45	216.4–376.7	37.90±3.15	13.36±1.13		
0.6	V_0	40	208.81±2.08	167.4–261.6	19.55±1.47	9.36±0.71	0.767±0.040	−0.246
	V_1		307.98±3.30	237.4–369.9	30.98±2.34	10.06±0.77		
0.5	V_0	34	213.98±3.89	172.7–270.6	22.69±1.86	10.60±0.88	0.792±0.043	−0.332
	V_1		356.34±4.31	294.9–451.5	37.26±3.04	10.46±0.87		

During the course of this inquiry into the osmotic behavior of the germinal vesicle of the *Arbacia* egg, a number of observations were made on the osmotic properties of the nucleolus as well. The nucleolus is a small spherical body, about 12 μ in diameter, appearing circular in optical section. It consists of a thick, highly refractive membrane enclosing a central lumen whose diameter is perhaps half that of the entire nucleolus. Less frequently the nucleolus has another form. It too is spherical, but smaller. The contents appear highly homogeneous, except for a few minute irrefractive "vesicles" enclosed within. It seems likely that this "solid" type of nucleolus is derived from the former by a rupture of the thick wall, followed by a loss of the contents of the large vacuole. The transformation is completed by a

TABLE VIII

Germinal vesicles of Arbacia punctulata

Relative Pressures	Number of Nuclei	Averaged Volumes ±P.E.$_M$ μ^3	Ranges μ^3	Standard Deviation, σ; ±P.E.$_\sigma$ μ^3	Coefficient of Variation, C; ±P.E.$_C$	Correlation Coefficient, $r_{V_0V_1}$; ±P.E.$_{r_{V_0V_1}}$	r_{zz}
		V_0 27.48±0.27	22.3–33.4	2.71±0.19	9.86±0.70		
0.8	46					0.838±0.030	−0.155
		V_1 34.38±0.36	27.4–44.9	3.67±0.26	10.67±0.76		
		V_0 27.52±0.37	19.0–36.1	3.95±0.26	14.35±0.98		
0.7	51					0.808±0.033	−0.275
		V_1 38.22±0.53	26.3–51.8	5.62±0.38	14.70±1.00		
		V_0 28.02±0.26	23.3–36.6	2.81±0.18	10.03±0.68		
0.6	53					0.667±0.051	−0.332
		V_1 43.97±0.44	33.8–55.5	4.75±0.31	10.80±0.72		
		V_0 28.75±0.36	20.6–37.6	3.75±0.26	13.04±0.91		
0.5	48					0.751±0.042	−0.435
		V_1 50.76±0.60	39.6–62.1	6.19±0.43	12.19±0.85		

refusion of the broken membrane, the entire process being accompanied by a general shrinking in volume. This phenomenon was actually observed in a swelling experiment with 80 per cent sea water. A few vesicles trapped within were the sole remnants of what had been the large central vacuole.

There is clear evidence that the nucleolus can swell and shrink reversibly. This is true whether one shrinks the cell in hypertonic solutions and then swells it back in 100 per cent sea water, or starts out by swelling it in diluted sea water and then shrinks it back in 100

TABLE IX

The estimation of equilibrium volumes, V_1, from initial volumes V_0

Relative Pressures	Nuclei		Cells	
	Regression Equation μ^3	Standard Error of Estimate* μ^3	Regression Equation μ^3	Standard Error of Estimate* μ^3
0.8	$V_1 = 3.18 + 1.14\ V_0$	±1.48	$V_1 = 14.55 + 1.13\ V_0$	±8.35
0.7	$V_1 = 6.62 + 1.15\ V_0$	±2.32	$V_1 = 11.85 + 1.25\ V_0$	±13.67
0.6	$V_1 = 12.37 + 1.13\ V_0$	±2.10	$V_1 = 53.98 + 1.22\ V_0$	±12.56
0.5	$V_1 = 15.18 + 1.24\ V_0$	±2.48	$V_1 = 78.34 + 1.30\ V_0$	±13.87

* Standard error of estimate, $\pm\sigma_{V_0}\sqrt{1 - r^2_{V_0V_1}}$.

per cent sea water. Just to give some idea of the magnitude of the changes that occur, three experiments with 60 per cent sea water will be presented; the figures should not be taken too seriously. Three cells whose nucleoli each had an initial volume of $0.8 \times 10^3\mu^3$ in 100 per cent sea water were transferred to 60 per cent sea water. After a half-hour in this solution, two of the nucleoli had attained the volume of $1.3 \times 10^3\mu^3$, and the other had increased in volume to $1.6 \times 10^3\mu^3$. A half-hour after restoration to 100 per cent sea water two of the nucleoli had returned to their initial volume of $0.8 \times 10^3\mu^3$ and the third had a volume of $0.9 \times 10^3\mu^3$.

Conclusions and Summary

1. The nucleus (germinal vesicle) of the immature *Arbacia* egg closely approximates an osmometer in its behavior. The nuclear membrane gives clear evidence of being almost perfectly semi-permeable and only deviates from the Boyle-van't Hoff law at the greater dilutions. Data have also been presented on the osmotic properties of the nucleolus and the cell as a whole. There is some evidence that in the process of growth both cells and nuclei tend to become less perfect in their osmotic behavior.

2. The germinal vesicle of the mature, unfertilized egg of *Nereis limbata* also behaves as an osmometer, though not as perfectly as that of the immature *Arbacia* egg. It seems significant that these experiments fail to reveal any appreciable amount of osmotically inactive material in either of the nuclei. In this respect, however, it appears highly desirable that new and independent methods of experimental attack be devised for the study of the problem of the "non-solvent volume."

3. Some attention has been paid, in the case of the germinal vesicle of the *Nereis* egg, to the analysis of the phenomenon of leakage and an experimental method was devised for studying this factor. It was found that, for dilutions of sea water not greater than 60 per cent the volumes attained by the nuclei at equilibrium are independent of the path traversed. In 50 per cent sea water, however, there is some evidence that this rule does not apply. The implication of these results is discussed with reference to the occurrence of leakage in hypotonic solutions.

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THE INFLUENCE OF THE SINUS GLANDS ON GASTROLITH FORMATION IN THE CRAYFISH

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Introduction

The sinus glands located within the eyestalks of crustaceans have long been known to have an endocrine function in regulating pigmentary effectors. These glands, first described by Hanström (1933), have been the subject of various studies. But even before the sinus glands were known, there was evidence that the eyestalks formed a chromatophore-activating substance.

Decrease of the intermoult period after eyestalk removal seems to be hormonal in nature although the evidence in support of this is not yet complete. Megušar (1912) found that ecdysis occurred at more frequent intervals in eyestalkless than in normal *Astacus*. Brown and Cunningham (1939) made similar observations on the crayfish, *Cambarus immunis,* and Abramowitz and Abramowitz (1940) and Kleinholz and Bourquin (1941) on *Uca pugilator*. Smith (1940) showed that removal of both eyestalks in young crayfish shortened the intermoult period by slightly more than 30 per cent. Darby (1938) states that operative injury hastens the onset of moult in *Crangon armillatus,* but Smith (1940) showed that this was not true in young *Cambarus clarkii*. Plankemann (1935) reported that such diverse factors as starvation, increased metabolism, color of background and pH of seawater might possibly affect the rate of moulting.

Since moulting and calcium metabolism are closely related phenomena, it might be expected that the mobilization and transport of calcium would also be regulated by a similar hormonal factor. Koller (1930) found that calcium in the moulted exoskeleton of *Crangon,* after eyestalk removal, was less than in the casts of normal animals and concluded that the so-called eyestalk hormone was definitely associated with calcium metabolism. Plankemann (1935) drew a similar conclusion from the known hormonal control of moulting in insects and the fact that the calcium content of the crustacean skeleton was modified during ecdysis.

Brown and Cunningham (1939) are the only ones so far to have tested the possibility of hormonal control of ecdysis in crustaceans by implantation of sinus glands after eyestalk removal. They found that the removal of the eyestalks increased the rate of moulting. When sinus glands were implanted into eyestalkless animals, there was a slight lengthening of the intermoult period. Low survival of their animals complicated their experiments so that while an endocrine factor in the regulation of ecdysis was indicated, conclusive evidence for the view was lacking.

In several of the Astacura gastroliths occur. They are rounded prominences, one on each side of the anterior region of the cardiac stomach, which are found in *Cambarus virilis* fully developed in the late spring.[1] The outer or lateral surface of each gastrolith is in contact with the epithelium of the stomach wall while the cuticular stomach lining covers the inner or medial surface. The outer surface of the gastrolith is smooth and convex, while the inner surface is flat.

Upon examination, a gastrolith can be seen to be composed of thin superimposed layers, the inner layers being parallel to the flat inner surface while the outer ones are concentric with the convex outer surface. A gastrolith is similar in structure to other parts of the exoskeleton except that the thicker layers are next to the epithelium.

During the moult the "stones" are freed by the casting off of the cuticular lining and are ground up and dissolved in the stomach, a new cuticle having been formed before shedding. According to Huxley (1889), in *Astacus fluviatilis* which are four years old, gastroliths begin to form forty days before ecdysis. The period is shorter in younger crayfish, being not more than ten days the first year after hatching. The process of their absorption takes twenty to thirty hours in young animals and seventy to eighty in the adult. Unless they are developed and then absorbed, ecdysis is not normally effected and the animal dies in the course of the process. Maluf (1940) states that gastroliths have about 3.16 per cent of the total ash present in the hard shell and are therefore of no significance after moulting. Numanoi (1939) describes the blood as more viscous and milky both during gastrolith formation and dissolution.

In an attempt to study the effect of the sinus glands on moulting in the crayfish, the gastroliths were most useful in determining oncoming moults, because their appearance precedes ecdysis and also since they are so easily detected by gross examination.

[1] Normal *Cambarus virilis*, collected in April and kept in the laboratory in running water, were observed to show a great burst of moulting in late May and early June. Of 75 of these animals, 38 moulted between May 27 and June 7.

The present investigation has been carried out with the hope of ascertaining more exactly the effect the sinus glands may have upon the process of ecdysis and especially of gastrolith formation in the crayfish. By the classical method of first removing the suspected gland and then reimplanting it, an attempt has been made to find what effect the gland has upon the formation of gastroliths which are indicative of oncoming moults.[2]

Material and Methods

In most instances observations were made on *Cambarus virilis;* however, *Cambarus clarkii* and *Cambarus immunis* were used in a few experiments. The specimens of *Cambarus virilis* were taken from local ponds and kept in running water in cement tanks except during experiments. The operated animals and controls were kept in some instances in individual finger bowls and in other instances in glass aquaria at room temperature (20–23° C.). The animals were not fed during experimental periods.

In removing the eyes, a thread was securely tied around the soft base of the eyestalk. With a small scalpel the part of the eyestalk just distal to the thread was severed. By this method bleeding was eliminated in most cases. At times some bleeding took place but a clot readily formed and the animals were not noticeably affected.

When a sinus gland was to be removed from an eyestalk in order to implant it into the same or another animal, the severed eyestalks were placed in Van Harreveld's (1936) crayfish perfusion fluid. The skeleton was split along the sides of the stalk by means of small bone forceps and the upper or dorsal part removed. This procedure exposed the sinus gland as a small bluish structure definitely differentiated from the mass of the eyestalk tissue. With the use of a dissecting microscope and fine needles it was relatively easy to disengage the gland and take it up in a pipette which was used in implanting the gland into the ventral abdominal region of the recipient animal.

In the histological study entire stomachs were removed and immersed in Bouin's fluid. They were rinsed in 70 per cent alcohol and then placed in 95 per cent and absolute alcohol. Cedarwood oil proved to be the best method of clearing before imbedding in paraffin. After sectioning they were stained with Mallory's triple stain and mounted.

Since the sinus glands, suspected of exerting hormonal control upon the moulting process in crayfish, are located in the eyestalks, the best

[2] This work was carried out under the direction of Dr. John H. Welsh, whose coöperation was most valuable.

approach seemed to be the removal of eyestalks from crayfish to determine whether their loss would cause the formation of gastroliths.

Eyestalk Ligation Experiments

In the first group of 29 *Cambarus virilis* both eyestalks were removed as mentioned above, the animals were killed after one or two weeks and their stomachs were examined. These experiments were started (using lots of 6 animals each) on September 28 and ran until December 7, 1940. In 25 of the 29 animals gastroliths were found, their size depending upon the weight of the animal and the length of time from operation to examination (Table I).

Table I

Animals with Two Eyestalks Removed

Eyes Off One Week	
Total number examined	12
Gastroliths present in	10
Average weight of animals	35.5 grams
Average weight of gastroliths (pair)	27.1 mg.
Eyes Off Two Weeks	
Total number examined	17
Gastroliths present in	15
Average weight of animals	19.9 grams
Average weight of gastroliths (pair)	10.1 mg.
Control Animals	
Total number examined	20
Number of animals found having gastroliths	none

In the second group one eyestalk was removed from each of 23 *Cambarus virilis* in the manner described above. These animals were killed after one or two weeks and their stomachs examined. In no instance was there any indication of gastroliths or signs that they were forming, as was the case in all normal controls. These experiments were started September 28 and ran through November 8, 1940.

Sinus Gland Transplantation Experiments

Since the removal of both eyestalks caused the animals to form gastroliths, it was decided to transplant sinus glands into eyestalkless crayfish to determine whether the glands might again exert their influence upon the animal's moulting process. If such were the case, these animals minus their eyestalks, and with implanted sinus glands should not form gastroliths.

In these experiments, all conducted between October 27, 1940 and March 7, 1941, the eyestalks were removed and two sinus glands implanted in the abdomen. In some instances the animals were examined one week after transplanting and in other instances additional transplants were made of two sinus glands at the beginning of the second week, and the animals were examined after two weeks. Twenty *Cambarus virilis* and 12 *Cambarus immunis* were used. Eleven of the animals died either on the day of the operation or the one following. Of those which survived, no traces of gastrolith formation were discernible upon examination of the stomachs.

Temperature Experiments

Through the fall and winter, in *Cambarus virilis*, eyestalk removal resulted in the formation of gastroliths. The question arose as to whether or not a low temperature would prevent gastrolith formation. It had been noted earlier by Smith (1940) that temperature had a marked influence on moulting in young eyestalkless crayfish (*Cambarus clarkii*).

Animals with their eyestalks removed were placed in aquaria in cold rooms set at various temperatures. Others were placed at room temperature (about 21° C.). They were examined after two or three weeks to determine the influence of temperature upon the formation of gastroliths. Tables II and III indicate the results.

In the experiments conducted in December and January minute gastroliths were found in only 5 out of 23 animals kept at 3° to 15° C., while all animals kept at room temperature had gastroliths at the end of two weeks. In the April–May experiments (Table III), due to the approaching spring moult, many of the animals had gastroliths present before eyestalk removal. (Assumed after examination of a number of normal crayfish most of which possessed small gastroliths.) Little, if any, increase in size of the gastroliths occurred, however, in the eyestalkless animals kept for three weeks at 3°, 5° and 15° C., while the crayfish which survived for three weeks at room temperature showed fully formed gastroliths. Thus it is seen that temperatures of 15° C. or lower prevent or at least slow up the formation of gastroliths induced by eyestalk removal. This suggests the possibility that an enzyme system is involved in the removal of calcium from the exoskeleton and its deposition as gastroliths and that this enzyme process is inhibited during the winter, in part by the low temperature, and in part by an inhibiting hormone from the sinus glands.

TABLE II

Showing animals with two eyestalks removed subjected to various temperatures (December–January)

Temperature	Animal	Experimental Period	Animal Weight	Gastroliths (pair) Weight
°C.		*in weeks*	*in grams*	*in mg.*
3	1	3	29.1	0
"	2	"	23.8	0
"	3	"	21.3	0
"	4	"	20.7	0
"	5	"	27.2	0
8	6	"	28.1	0
"	7	"	24.2	2.2
"	8	"	27.0	0
"	9	"	27.2	0
"	10	"	38.0	2.8
"	11	"	26.0	0.6
11	12	2	14.1	0.2
"	13	"	17.5	0
"	14	"	17.1	0.2
"	15	"	13.8	0
"	16	"	14.0	0
"	17	"	23.3	0
15	18	"	24.9	0
"	19	"	21.3	0
"	20	"	20.7	0
"	21	"	23.1	0
"	22	"	17.6	0
"	23	"	16.8	0
20–23	24	"	24.1	9.6
"	25	"	29.3	0.9
"	26	"	27.2	20.6
"	27	"	19.5	3.0
"	28	"	21.3	21.4
"	29	"	25.9	20.0
"	30	"	18.2	52.9
"	31	"	26.4	57.0
"	32	"	21.5	7.4
"	33	"	28.3	20.2
"	34	"	34.3	0.4
"	35	"	23.5	7.8
"	36	"	29.2	9.3
"	37	"	34.5	9.2
"	38	"	3.9	37.6
"	39	"	22.2	21.3
"	40	"	4.0	21.2

Histological Study

When it was seen that gastroliths were formed prior to moulting, whether this moulting were normal or induced by eyestalk removal, the question of the histological changes in the stomach epithelium prior to, and during gastrolith formation, arose. How soon after removal of

Table III

Showing animals with two eyestalks removed subjected to various temperatures (April–May)

Temperature	Animal	Experimental Period	Animal Weight	Gastroliths (pair) Weight
°C.		*in weeks*	*in grams*	*in mg.*
3	1	3	20.5	1.1
"	2	"	13.2	26.2
"	3	"	19.0	6.0
"	4	"	16.4	9.2
"	5	"	15.2	5.2
"	6	"	19.0	0
"	7	"	18.5	3.0
"	8	"	13.4	0.4
5	9	"	20.4	0
"	10	"	20.0	4.3
"	11	"	21.0	0.2
"	12	"	14.7	0
"	13	"	19.6	0
"	14	"	22.0	1.3
"	15	"	16.4	6.2
"	16	"	13.0	17.0
15	17	"	19.5	0.2
"	18	"	10.5	4.2
"	19	"	14.2	8.1
"	20	"	14.9	17.0
"	21	"	15.0	2.3
"	22	"	12.5	0
"	23	"	13.2	5.0
"	24	"	12.0	4.0
20–23	25	"	12.0	199.0
"	*26	"	11.0	141.0

* Four animals in this group died before the end of three weeks.

sinus glands is there a noticeable change in the stomach epithelium which secretes the gastroliths? What are these changes?

Regions of the stomach where gastroliths form were fixed and studied histologically. Figure 1 indicates the condition of the stomach lacking gastroliths. Figure 2 illustrates the epithelial changes observa-

ble in the same region when a gastrolith is starting to form. Twenty-four hours after eyestalk removal there is proliferation of the epithelial cells (Fig. 2) which were originally of a low columnar type (Fig. 1). Gradually these cells become tall columnar cells. There is a subsequent crowding of the sub-epithelial connective tissue. By the end of 48 hours the cells are tall, crowded together and show large prominent nuclei (Fig. 3). Sections made of stomachs of animals whose eyes had been removed for one or two weeks show much the same condition of the

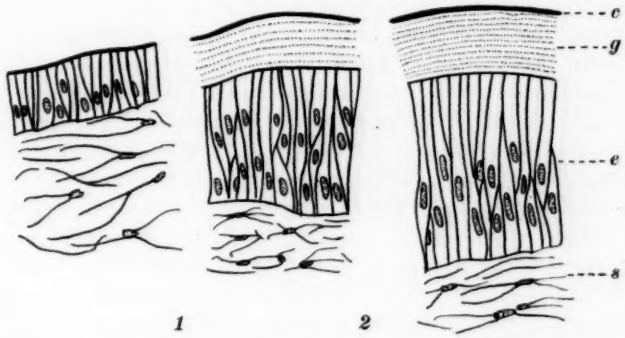

FIG. 1. Section of normal stomach of *Cambarus virilis* in region where gastrolith will form.

FIG. 2. Section showing gastrolith formation in stomach of *Cambarus virilis* 24 hours after eyestalk removal.

FIG. 3. Section showing gastrolith formation in stomach of *Cambarus virilis* 48 hours after eyestalk removal.

Abbreviations: *C*, cuticle; *G*, gastrolith; *E*, epithelium; *S*, subepithelial connective tissue.

epithelium as is illustrated by Fig. 3. Gastroliths begin to form 24 hours after eyestalk removal. However, Bouin's fluid containing acetic acid dissolves much of the inorganic material of the gastroliths and makes a true histological picture difficult to reconstruct.

DISCUSSION

During the fall and winter normal animals show no gastroliths. Induced gastroliths, that is, those formed after eyestalk extirpation, were never found to be as large as those present in the spring and early summer during the normal moulting period. This was apparently due in part to the fact that gastroliths are from three to four weeks in their formation (forming much of their thickness during the last week) while

the experimental eyestalkless animals were ordinarily killed after a period of one or two weeks.

According to Robertson (1941) members of the suborders Anomura and Astacura do not appear to store calcium in the hepatopancreas. In Astacura gastroliths are formed in the foregut prior to moult. The gastroliths represent material which has been resorbed from the skeleton. A few hours after moulting they decrease in size and finally disappear, apparently being used as a source of calcium for the new skeleton.

Several investigators have studied the relation between calcium and moulting in Crustacea. The gastroliths, when present, and hepatopancreas, have been suspected as internal reserves drawn upon as the new skeleton is formed. Hecht (1914) found that once the skeleton of *Callinectes* was cast, the amount of calcium in the new individual was insufficient for the rebuilding of the new skeleton. Kleinholz, with the assistance of Emma Bourquin (1941), found that *Uca pugilator,* killed within five minutes after ecdysis, contained calcium equal to 1 per cent of the dry body-weight. The internal reserve constituted only 6 per cent of the total calcium content of the normal intermoult crab. The animal absorbed the balance of its calcium after moulting. Paul and Sharpe (1916) reported that in decapod Crustacea calcium was withdrawn from the hepatopancreas after ecdysis, and after the new shell was hardened, calcium was almost absent from this gland. Numanoi (1939) found the gastroliths of *Sesarma haematocheir* enlarged as moulting approached and disappeared after the moult. These changes he observed to take place concurrent with periodic changes in the blood calcium level.

Apparently the sinus gland acts as an agent controlling the passage of calcium, previous to moulting, from the exoskeleton into the blood where some of it is conveyed and deposited in the forming gastroliths. These act as reserve calcium which is utilized by the animal in order to harden partially its new skeleton until it can absorb more from its food and surroundings. Numanoi (1939) found that, in a species of *Sesarma,* when the calcium reserve is being dissolved after moulting, prior to being reprecipitated in the integument, blood calcium is raised to over forty times its normal level.

Why the sinus glands, which during the fall and winter inhibit moulting, do not do so in the spring is an interesting question. Apparently there is a seasonal variation in their activity which in the spring may be checked or inhibited by some other mechanism; also the low winter temperature would play some part in inhibiting gastrolith formation.

That the sinus glands are concerned in calcium metabolism of the crayfish during its moulting activity seems now to be reasonably certain.

The close relationship between the transfer of reserve calcium and moulting tends to point toward one mechanism as a control of both processes. The more intimate details of this mechanism still remain to be worked out.

Summary

1. Removal of both eyestalks of *Cambarus virilis* and *Cambarus immunis* induces the formation of gastroliths at times of the year when they would not normally form. Removal of one eyestalk fails to induce gastrolith formation.

2. Transplantation of sinus glands into the abdominal region of eyestalkless crayfish prevents the formation of gastroliths.

3. Temperatures of 15° C. and lower prevent or greatly retard the formation of gastroliths in eyestalkless crayfish.

4. As early as twenty-four hours after eyestalk removal marked histological changes occur in the regions of the stomach which secrete the gastroliths. There is proliferation and increase in height of the epithelial cells and they have already begun the secretion of the gastroliths.

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THE CYTOLOGY OF THE ANTERIOR PITUITARY OF THE FOWL

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Introductory Statement

This paper is, for the most part, concerned with cytological questions, but it also attempts a correlation of cytological facts with experimental data. The study is by no means completed and will be carried on with the thought that an exhaustive study of one form is very much needed. The experimental side of the work has been done by Dr. W. R. Breneman and due recognition is here given for that help. Without it, this study could not have been as extensive as it has been.

In a study of this kind, it is essential to know what happens in the normal course of events insofar as can be determined. These changes in themselves are interesting, but such knowledge becomes necessary as a basis for interpreting experimental results. One of the difficulties of the problem is in recognizing what the normal course of events is, for there is much variation due to variable and uncontrollable external and internal factors. This difficulty, however, can be overcome in part by the study of many pituitaries and this has been done (more than 800).

The White Leghorn chick has served as the basic material. Other varieties have been used to some extent for supplementary and comparative purposes, and where they are discussed in the text the fact will be made clear at that place. Severinghaus' modification of Nassonov has proved to be the best for cytological details. Nassonov and Helly were also used as well as Bouin. Various ways of staining were employed but the acid fuchsin methyl green method of Severinghaus gave the best results for most purposes. Other stains were used largely as checks. For a study of the Golgi material the usual osmic treatment was applied.

Development and Morphology

On the bases of the distribution of the cells and the shape of the gland, the fully differentiated anterior pituitary has been described as divided into two parts. Rahn (1939), in particular, has accumulated considerable evidence in favor of this point of view and speaks of these

[1] Contribution No. 300 from the Zoölogical Laboratory.

two parts as the cephalic and caudal lobes. By some observers, including Rahn, this differentiation is carried back into the embryo as far as the sixth day. My own findings do not support this latter conclusion. The constrictions seen in the six-day embryonic gland are nothing more than irregular foldings of the wall of the oral evagination and may occur near the middle or anywhere else (Fig. 3, Plate I). At nine and fourteen days (Figs. 2 and 4, Plate I) there is even less evidence of a division. It is true that in the pituitary of fowls and of birds in general the acidophiles are usually distributed over only about half of the gland and when so distributed the two ends of the gland are very different in appearance. In some pituitaries, however, particularly from birds two to three months old, the acidophiles may extend over two-thirds or three-fourths (Fig. 5, Plate IV) of the entire gland and in such cases the lobe-like appearance is absent. This statement does not apply to a few small scattered cells, but to numerous large functional acidophiles.

Controls

My studies of the control chick pituitaries (post hatching) have included the 3, 10, 15, 20, 25, 30, 35, 40 and 56-day periods. Some observations have been made at still later stages, but such studies are incomplete and must be supplemented with more material of known ages.

At three days the pituitary seems to be made up wholly of acidophiles and chromophobes. I say seems to be, for if any basophiles in early stages of transition are present, they cannot be sharply separated from the chromophobes and the non-granular acidophiles. Transitional stages, however, must be present, for functional basophiles are found at ten days. Rahn describes them in late embryonic development but I cannot confirm his observations.

Considerable work has been done in trying to discover one or more diagnostic characters by which cell types can be distinguished in early development. Size certainly does not answer the purpose in the fowl

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Plate I

Figs. 1, 2, 3 and 4. Sagittal sections of embryonic pituitaries 3, 9, 8 and 14 days. In Fig. 2, the cavity of the hypophysial stalk has disappeared and in Fig. 4 the stalk itself has regressed in places. Note the place of attachment of the stalk in all figures. The posterior pituitary is omitted in Fig. 4.

Fig. 5. Sketch under low power, of a small area of a pituitary from an old fowl. The circles are outlines of the large golden spheres which appear in degenerating basophiles. The larger spheres are larger than the cells which means that the cells have broken down. Cell outlines are not shown. The drawing demonstrates particularly the large number of these spheres.

Fig. 6. A low-power sketch of a few of the cords with acini in a five-year-old Rhode Island Red cock. These acini appear shortly after two years of age in Barred Rock hens and become more numerous with age.

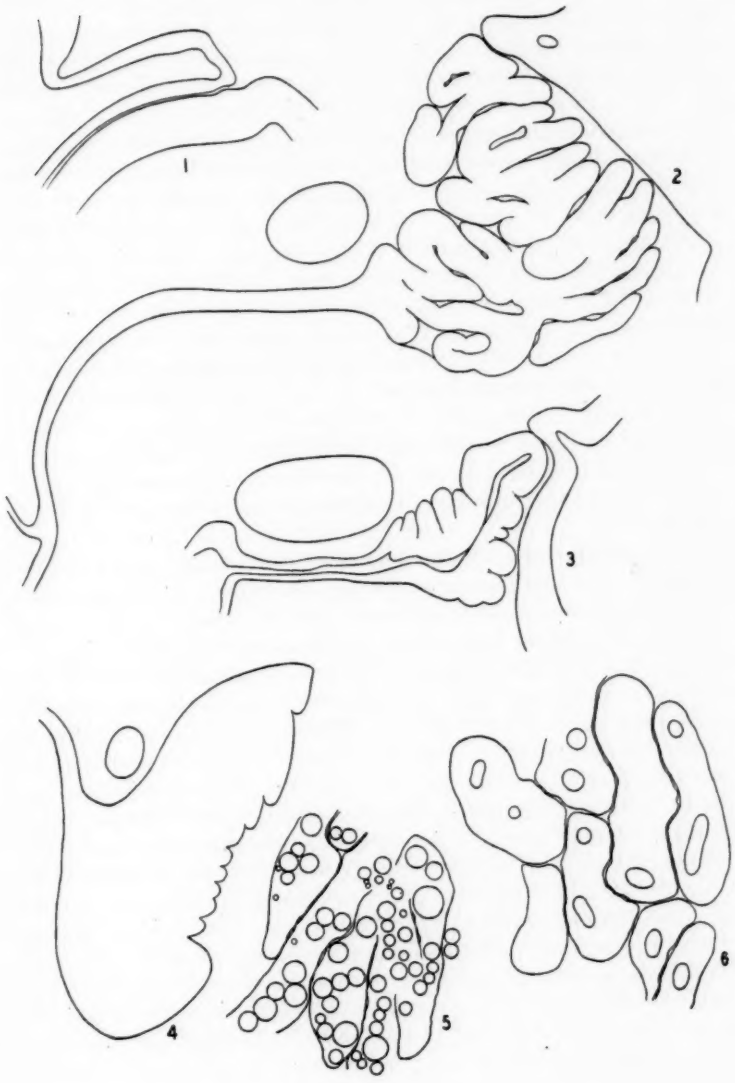

PLATE I

even though acidophiles and basophiles do in the end become larger than chromophobes. Neither does the shape of the cell, the shape and position of the Golgi material, the position of the nucleus, nor the position of the cell lend any help. In the large functional basophiles there are one or two (sometimes three) large spherical nucleoli which may appear vacuolated. The chromatin seldom is visible and if so, only faintly. This condition is in sharp contrast to that found in functional acidophiles where there are two or more small nucleoli, irregular in outline, and often somewhat obscured by irregular flocculent masses of chromatin. These two kinds of cells, then, as they approach the functional stage, can be distinguished fairly accurately on the basis of nuclear appearance. The nuclear picture in the chromophobes is very similar to that of the acidophiles. Unfortunately, transitional stages between the chromophobes and chromophiles cannot be clearly distinguished, probably due to the fact that changes are gradual. Staining reactions of nucleoli, nuclei, and cytoplasm help very little or none previous to the time of formation of secretory granules. In the ten-day embryo, Rahn distinguishes transitional cells on the basis of the degree of staining, the basophiles being much darker, but it seems to me such a method is very uncertain.

At three days the acidophiles are distributed throughout the pituitary but they are not alike in the two ends of the gland. In the caudal lobe, using Rahn's terminology, the cells are larger and possibly more columnar, although a conspicuous columnar arrangement does not enter until much later. As a rule, they stain more intensely, but this may be due to a greater accumulation of secretory granules. In fact, on the same slide I have seen cells in the cephalic end a brilliant carmine while those in the caudal end were orange. With the Altmann stain there is considerable variation in color, even in the same lobe when different slides are compared. Making allowance for all these variations, and for the fact that the caudal end cells may extend far into the cephalic region, the cells in the two ends still show differences. On the average, the cephalic end cells are smaller. They are more sparsely granulated and the granules may stain purple similar to the mitochondria. Differences in the two ends are particularly striking following fixation in Helly but Helly is not a good cytological fixative. Unfortunately Dawson and Friedgood's method (1937) by which they demonstrated two types of acidophiles in the rabbit and cat did not prove to be helpful, probably due to lack of skill on my part. Rahn definitely commits himself in favor of the point of view that there are two kinds of acidophiles, and on the basis of present evidence, I think this interpretation is correct. I shall refer to the caudal acidophiles as A1, the cephalic as A2.

Even at three days and probably at all later stages, there are cells with pycnotic nuclei. The degree of pycnosis is variable, grading from nuclei with only slight changes to others in which no structures are visible. Accompanying the pycnosis is a reduction in the size of the nucleus. The entire cell also becomes pycnotic, smaller in size, and modified as to shape (Figs. 6 and 7, Plate II). Are these pycnotic cells special cells which have developed directly from the chromophobes and if so, what could their function be? If they are not special cells, and I do not think they are, they must, in early post-hatching stages, be modified acidophiles for the basophiles remain undeveloped at three days. That these cells are modified acidophiles is clearly indicated by the fact that some of them have secretory granules in the cytoplasm. In later stages, after the basophiles become functional, they too may change in the same way (Figs. 2 to 5 and 8, Plate II).

Rahn describes pycnotic cells in the adult pituitary of the chick and identifies them as modified acidophiles. He says the greater and denser the amount of granulation, the darker and more pycnotic is the nucleus. My own observations show clearly that cells which exhibit the highest degree of pycnosis are completely or almost completely degranulated. They are cells which are regressing, probably toward chromophobes, possibly toward complete inactivity. In castrates where regressing acidophiles can be identified more accurately, pycnosis does not become so acute. My own point of view for the present is that cells with pycnotic nuclei as well as cells with pycnotic cytoplasm are acidophiles and basophiles which have passed the peak of activity. Speaking generally, active cells are not pycnotic, either with respect to the nucleus or the cytoplasm.

Ten-day Males

At ten days the acidophiles and chromophobes are still dominant. The general appearance and distribution of these cells have not changed greatly over the three-day period. The gland is larger, due in part to cell multiplication, and in part to cell enlargement. The major change at the ten-day period is the presence of large basophiles which are, without doubt, in the functional secretory stage. This is contrary to Rahn's statement that the basophiles are not functional until three weeks after hatching, but the evidence is clear and convincing. Besides, my observations are supported by the experimental evidence of Breneman (1937), who has shown by studies on gonad and comb growth that the testes have enlarged and that the male sex hormone is being secreted between ten and fifteen days. The basophiles, at this time, approach maximum size (see Fig. 1, *a* to *h*, plate IV, for comparative sizes). The

nucleus is large and spherical and the nucleolus (sometimes two) has become large in comparison to its former size. The cytoplasm and nucleus both stain well with methyl green. The cytoplasm, in different cells, varies somewhat in appearance. Sometimes it is uniform, sometimes slightly flocculent, and sometimes definitely granular. My conclusion that the basophiles are functional at ten days is based upon the fact that these cells are similar to functional basophiles in later stages of development as well as Breneman's experimental evidence. These functional basophiles occupy an area not definitely delimited and which can best be described as the central juxta-neural region, a region extending into both ends of the pituitary. More large basophiles are found in the caudal end, however, than in the cephalic. See Table I for body, comb, and gonad weights of 10-day control chicks.

Ten-day Female

As in the male, functional basophiles are present in the ten-day female. The general distribution of all cells in the two sexes is about the same, but the size of the gland in the female is slightly smaller. This difference may be due to slower division rate or again to fewer large basophiles. In some glands it appears as if the acidophiles were actually

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PLATE II

FIG. 1. Outline sketch of a basophile from a 20-day control male.

FIGS. 2, 3, 4, 5, 6, 7, 8. Cells showing a series of retrogressive stages in both basophiles and acidophiles. Figs. 2, 3 and 8 are basophiles; Figs. 6 and 7 are acidophiles; Figs. 4 and 5 cannot be identified with certainty.

FIG. 9. An acidophile from a 30-day limited diet female showing a cluster of green granules adjacent to the nucleus.

FIG. 10. An acidophile from a 30-day limited diet male showing green granules but more scattered than in Fig. 9.

FIG. 11. A much modified basophile from an old (age unknown) Barred Rock cock. The cell is filled with spheres, which, following the acid fuchsin methyl green staining method, are golden yellow with a red periphery. The nucleus is dark and compressed.

FIG. 12. An acidophile in division.

FIG. 13. A basophile from a Buff Leghorn capon about seven months old. The numerous dark bodies in the cytoplasm are mitochondria.

FIG. 14. A basophile from an eight-week-old White Leghorn capon. Note the three large nucleoli.

FIG. 15. Basophile from an old White Rock hen. The dark spheres in the cytoplasm are mitochondria. The light spheres are golden yellow as in Fig. 11.

FIG. 16. A much modified basophile from a five-year-old Rhode Island Red cock. The large central sphere is golden yellow with a red periphery, similar to the smaller spheres in Figs. 15 and 16. The nucleus is pushed to the periphery of the cell.

FIG. 17. A basophile from a Barred Rock male about three months old. The golden bodies appear early in this bird and are irregular in outline instead of spherical as in Fig. 15.

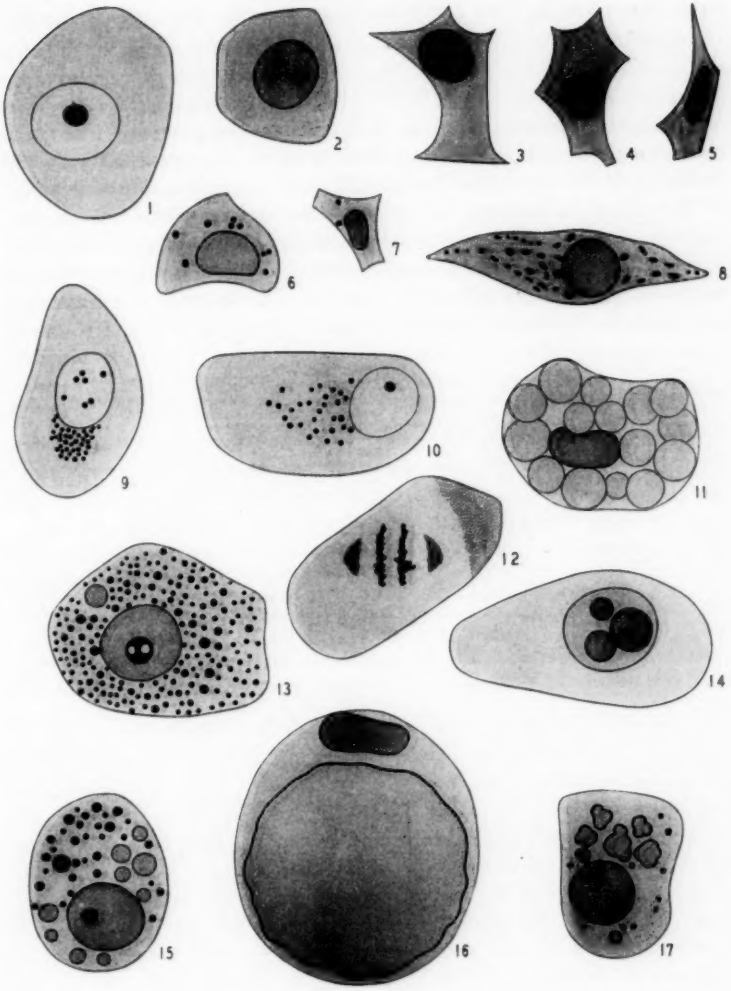


PLATE II

more numerous and even larger and more granular than in the male, but the evidence is not convincing.

Fifteen days and Older

Changes in the pituitary from 10 to 56 days are gradual. The gland increases in size due to the multiplication of all three types of cells and to the growth of the chromophiles. The rate of increase in the numbers of the cell types, however, is not the same, and hence the relative proportions change. While cell counts have not been attempted, the statements which follow are reasonably accurate, perhaps as accurate as they would have been had cells been counted. At 10 days, as stated, the chromophobes and acidophiles are the dominant types. Only a relatively

TABLE I

Body, comb, gonad weights and pituitary size in controls

Age	Body Weight	Comb Weight	Gonad Weight	Comb Weight	Gonad Weight	Pituitary Size[3]
days	*grams*	*mg.*	*mg.*	*per cent of body weight*	*per cent of body weight*	
10	76.8	34.8	19.5	0.045	0.0254	2672.
15	106.0	115.4	36.9	0.109	0.0349	3378.
20	175.8	229.4	52.3	0.164	0.0291	4715.
25	190.7	316.6	61.0	0.166	0.0320	5302.
30	272.3	1336.9	86.0	0.491	0.0316	5958.
40	406.5	3740.0	179.7	0.922	0.0442	

[3] See footnote 7, Table II.

few basophiles are present but after 10 days the basophiles increase in number more rapidly than the acidophiles. The relative proportions of basophiles and acidophiles, however, vary widely with variable external conditions such as food, living conditions, etc. Speaking generally, adverse conditions restrict basophilic development but have little effect upon the acidophiles. Sometimes the acidophiles actually stand out more prominently, but this impression may be due to the contrast with the small number of basophiles. Certainly the acidophiles are large and granular and the number is not decreased when chicks are reared under unfavorable conditions. One is tempted to suggest here that if the acidophiles secrete a growth-promoting hormone, they are secreting more actively to overcome the handicap of a poor environment.

After making allowances for variations, both external and internal, the basophiles increase in number as development progresses and under favorable conditions they become more numerous than the acidophiles at 56 days of age and probably earlier in some chicks. The exact relative

proportions, however, are not important. The fact which is important is that both kinds of cells are functioning and that the basophiles increase in number from the 10-day stage. During the laying periods of hens one and one-half years and older, the basophiles are the dominant cell type. As previously stated, functional basophiles appear first in the central juxta-neural area about ten days after hatching. From there they spread gradually into the posterior end and later (30 to 40 days old) into the anterior end. By 56 days they are found in all regions.

In Rhode Island Reds and Barred Plymouth Rocks no functional basophiles were present in the pituitaries of 20-day-old chicks. Many basophiles were present, however, in 25-day Barred Rocks. Twenty-five-day Rhode Island Reds were not studied. Here also we find a correlation between cytological observations and experimental data. Breneman (1941*b* and unpublished data) finds very little or no indication of sex hormones present in these two varieties at 20 days of age. These observations, in addition to the fact that Rhode Island Reds age earlier than White Leghorns, demonstrate clearly that varietal differences with respect to pituitary behavior exist.

Comparative pituitary sizes are of interest, particularly since they are in harmony with cell changes and with comb and gonad growth. See Table I.

Golgi Substance and Mitochondria

As many biologists accept the theory that the Golgi substance is concerned with secretion and since Severinghaus has used the shape, size and position of this material as a means of identifying the acidophiles and basophiles, I wish to comment briefly on the behavior and significance of this substance as I have observed it in the fowl. My studies have been limited to osmic-treated pituitaries. Severinghaus has described two distinct types of Golgi substance or apparatus in the rat. In one type the substance "consists of a basket-like network closely applied to one side of the nucleus." The second type is somewhat spherical in shape, sometimes with one or two invaginations, and lies in the cytoplasm separated from the nucleus. The first type is characteristic of acidophiles, the second type of basophiles. The two types can be distinguished in the chromophobes.

In the fowl, unfortunately, the Golgi substance cannot be used as a distinguishing mark for the acidophiles and basophiles. An examination of this substance in the chromophobes such as those illustrated in Plate III shows clearly that there is much variation in form, shape, size and position. Even in granular basophiles and acidophiles, where there is no doubt of their identity, I cannot say that I can always identify these

cells by means of the Golgi substance. As the substance is difficult to describe with words, I prefer to let the figures, which have been carefully and accurately drawn to the same scale, speak for themselves. In Fig. 1, Plate III, is shown the Golgi substance in a large functional basophile and this appearance is general, except that in many of these cells, even after prolonged osmication, the substance is only faintly visible or cannot be seen. Figure 3, Plate III, shows an acidophile in which the Golgi substance is very similar in shape and position to that in Fig. 1. The osmication, however, is more pronounced. In other acidophiles, as illustrated in Figs. 4, 5, and 6, the substance is net-like. I have never seen this form in a functional basophile. In most or all of the columnar acidophiles the Golgi substance lies at the inner end, the end distal to the capillary. Apparently there is no orientation in the basophiles as illustrated in Fig. 3, Plate IV.

The presence of the Golgi substance in secretory and many other kinds of cells has raised questions no one of which has been answered with any degree of satisfaction. There are so few facts of which we are certain; of theories there are plenty. This is not the place to discuss these questions. I do wish to remark, however, that I find no convincing evidence that the Golgi substance is directly or even indirectly concerned with secretion. Secretory granules, so far as I can discover, may appear in regions of the cell remote from the Golgi substance.

PLATE III

With the exceptions of Figs. 2 and 17, all figures on the plate are from osmicated pituitaries and with the exception of Figs. 2, 7, and 17 were drawn to show size, shape, position and morphology of the Golgi substance in the cells of the anterior pituitary of the White Leghorn fowl. Most of the figures are from 13- and 18-day embryos and the cells drawn were selected at random, without regard to kind, in order to show as much variation as possible in the Golgi substance.

FIG. 1. A large basophile from a 47-day capon castrated at five days. There is very little accumulation of osmic. Hence the Golgi substance shows only faintly.

FIG. 2. A large basophile in a 46-day castrate which was injected daily from the thirty-fifth to the forty-fifth days with 0.5 mg. Oreton B. Note irregular shape of nucleolus.

FIGS. 3, 4, 5, and 6. Acidophiles. Figure 3 from a male 31 days old; Fig. 5 from a young male of unknown age, weight 2.5 lbs.; Figs. 4 and 6 from a female 45 days old. Note the net-like appearance of the Golgi substance in Figs. 4, 5 and 6.

FIG. 7. Acidophile from a 45-day female. Pituitary was treated with osmic. In the distal end of the cell (adjacent to blood vessel) is a large spherical gray mass with a smaller black sphere near the center. In Fig. 4 the same body is present but the gray sphere was not visible and the black sphere is in the form of a ring.

FIGS. 8, 9, 10, 12, 13, 15, 18, 19, 20, 21, 22, 23. Cells from an 18-day embryo Drawn to show Golgi substance. Type of cells not identified. Most are evidently chromophobes; some are probably acidophiles.

FIGS. 11 and 14. Cells from a 13-day embryo. Golgi as in 18-day embryo.

FIG. 16. Chromophobe from a young male of unknown age. Weight 2.5 lbs.

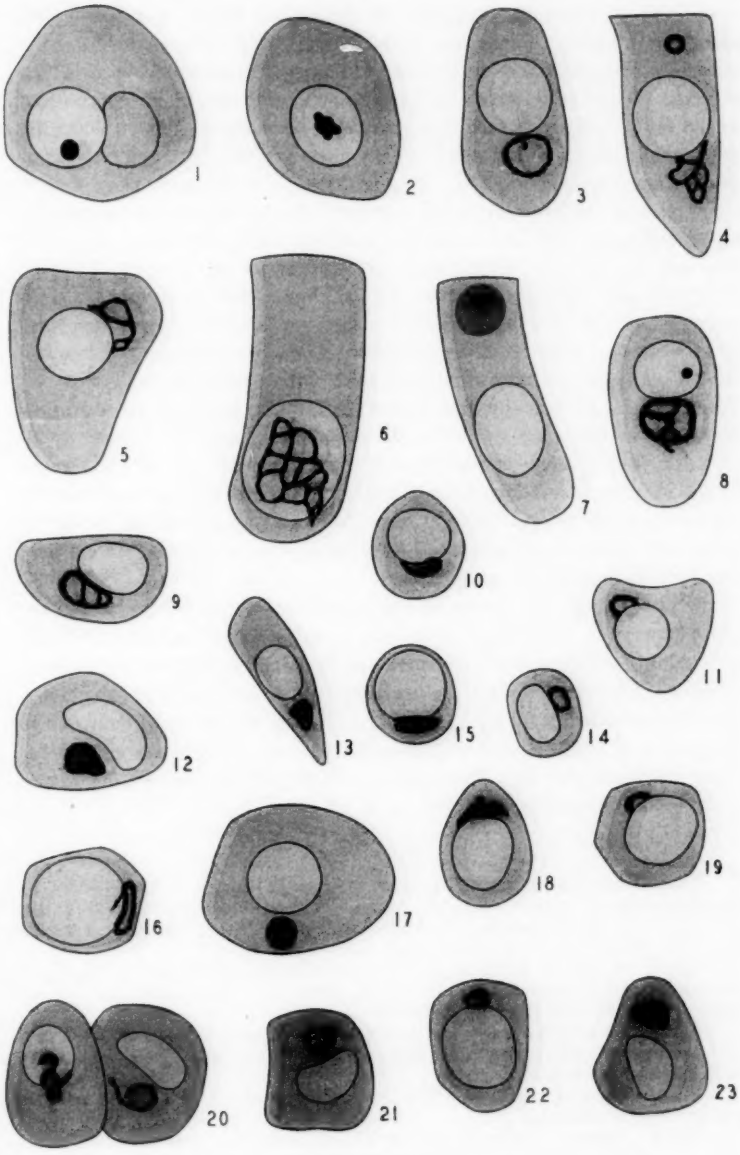


PLATE III

Figure 9, Plate II, illustrates an acidophile which was filled with secretory granules (not shown in the sketch). In addition to the usual secretory granules there is a cluster of larger granules lying near the nucleus. They stain green or bluish-green and are in sharp contrast to the red or yellowish secretory granules, when the acid fuchsin methyl green staining method is used. They may migrate into other parts of the cell as shown in Fig. 10. The position of the cluster, as in Fig. 9, indicates that the granules occupy or may occupy the same region as the Golgi substance. What are these granules? The first thought is that they are mitochondria for in other cells such as young insect ova (Payne, 1932) the mitochondria and the Golgi substance may in certain developmental stages be mingled together in a nuclear cap area (metabolic center of Schooley). Against this possibility, however, is the fact that mitochondria should, with the technique used, stain a purplish red. Further, mitochondria are usually distributed at random and can be definitely identified in the same cell with the green granules. Of course, mitochondria may change chemically and react differently at different times to the same stain. More definite statements about these green granules cannot be made at this time. I have, however, kept in mind the possibility that both acidophiles and basophiles may synthesize more than one kind of secretory granule.

Very little additional need be said about the mitochondria. They are more numerous in the basophiles than in the acidophiles, are distributed at random, and seem most numerous in the large basophiles of castrates (Fig. 13, Plate II). They are usually granular and variable in size, but may appear vesicular (Fig. 15, Plate II). In retrogressing cells, as in Figs. 2 to 8, Plate II, the mitochondria assume variable shapes and become less numerous with diminution in the size of the cells. No evidence as to their function has been obtained.

PLATE IV

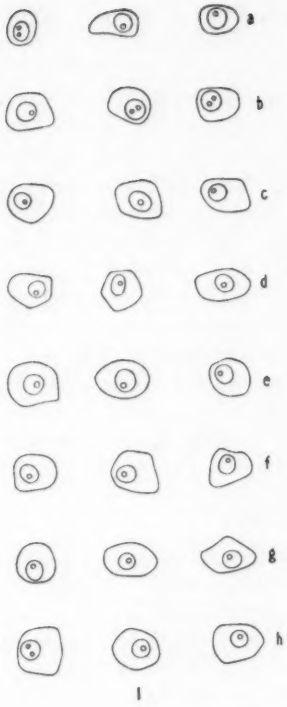
FIG. 1. A series of drawings to show the relative sizes of the large basophiles at different ages in control males and females. Drawn to the same scale. (*a*) 10-day male; (*b*) 10-day female; (*c*) 15-day male; (*d*) 15-day female; (*e*) 20-day male; (*f*) 25-day male; (*g*) 30-day male; (*h*) 56-day male.

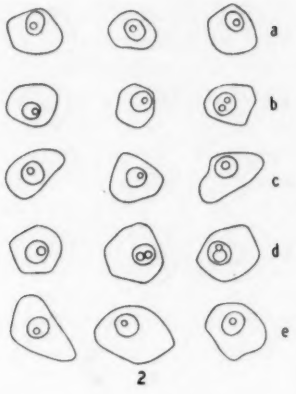
FIG. 2. A similar series of drawings to show basophilic size in castrates. Drawn to same scale as Fig. 1. (*a*) 20-day male; (*b*) 30-day male; (*c*) 35-day male; (*d*) 56-day male; (*e*) 90-day female.

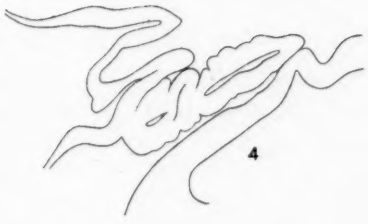
FIG. 3. Basophiles from 47-day castrate male. Drawn to show shape of cells and position of nucleus and Golgi substance within the cells. Note irregularity with respect to these points.

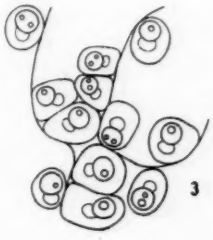
FIG. 4. Saggital section of a 5-day embryonic pituitary. Note folds and secondary oral evaginations.

FIG. 5. Saggital section through middle of pituitary of male chick 10 weeks old. The dotted line gives the boundary between two types of acidophiles.









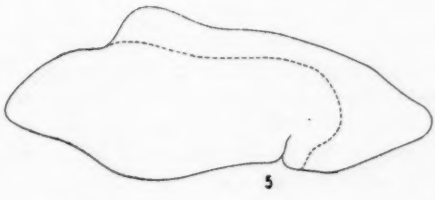


PLATE IV

An Unknown Structure

In one female 45 days old, the pituitary of which had been osmicated, an unusual structure was found in many of the acidophiles. Other than in this one pituitary it has not been seen. The structure (Figs. 4 and 7, Plate III) which is sometimes double appears as a black ring or sphere lying within a dark gray more or less spherical area, the whole of which lies in the peripheral end (toward the blood vessel) of the columnar-shaped acidophile. Since the Golgi substance lies in the opposite end of the cell and since there is no connection between the two, it seems independent of the Golgi substance. Neither does there seem to be any possible mitchondrial connection. In some cells the gray area does not show, due probably to uneven osmication or to uneven extraction of the osmic. Such irregularities in osmicated material are common.

A single instance of this kind might lead one to think that the structure is an artifact, but this does not seem possible. More likely it is a structure which becomes visible only when the cytological treatment, including osmication, is favorable. As to what possible function it might have, I make no suggestions.

Limited Diet Birds

Breneman (1941*a* and 1941*b*) has performed several experiments with limited diet birds. We mean by "limited diet" a limitation in quantity of food. After hatching, the chicks were fed on the second and on alternate days. In some experiments with older birds, food restriction was even more drastic. As the intake varied in different individuals, the responses to the limited diet treatment were variable. Pituitaries from 15-, 20-, 30-, and 31-day limited diet chicks have been studied, and also pituitaries from 15- and 30-day limited diet castrates. In all the limited diet non-castrate chicks the general effects are the same. The basophiles are undeveloped or restricted in comparison with the normal diet controls, and the acidophiles are as numerous and as well developed as in the controls. They actually seem, in some instances, to be more numerous than in controls but the different appearance is probably due to the changed proportions of the two types of cells. The pituitaries of the limited diet chicks are, without exception, smaller than in the normal diet controls. For relative sizes see Table II.

According to Breneman's data (1941*b*), the body, comb, and gonad weights of limited diet male chicks are much less than in controls (Table III). Since the basophiles of the pituitary are greatly restricted in their development in limited diet chicks, we have a direct correlation between the low gonad weight and the meagre development of the

basophiles. The natural inference is that in limited diet chicks the gonads remain small, in part, at least, because of the limited amount of gonadotropic hormone present.

In contrast to the low weight of comb and gonads in limited diet chicks, Breneman (1941*b*) found the intestine longer and heavier in

TABLE II

Size[7] relations of pituitaries (from males)

Age	Controls	+5 I.U. Estrone	+100 I.U. Estrone	+500 I.U. Estrone	+0.0005 mg. Stilbestrol	+0.2 mg. Stilbestrol	+0.125 mg. O.B	+.01 mg. O.B	+2 units Prolactin
20 day emb.	1860								
3 day	2068								
10	2672.5								
15	3378.5	2740		2415					
20	4715		3223	3696.6			4216.6		3552.5
25	5302								
30	5958.3	4362.5		3926.6	4020	3525		4970[4]	
35	6512.5								
56	9400								

Age	Castrates	Castrate +100 I.U. Estrone	Castrate +5 I.U. Estrone	Castrate +0.125 mg. O.B	Castrate +0.05 mg. O.B[5]
20 day	5376.3	4843.3		4120	
25	6105				
30	7084		6205		6460
35	7540				
56	13832.5				

Age	Limited diet	L.D.[6] Castrate	L.D. +500 I.U. Estrone	L.D. ♂ +0.01 mg. O.B	L.D. ♀ +0.01 mg. O.B	L.D. Castrate +0.01 mg. O.B	L.D. +5 I.U. Prolactin	L.D. +0.07 mg. O.B	L.D. +pellets T.P.
15 day	2095.6	2055		2760	2358	2527		2843.3	2053.3
20	3110		2595				3600		
30	4288	5610							

[4] Thirty-one days old.

[5] Oreton B (dihydroandrosterone benzoate).

[6] Limited diet.

[7] For comparing gland size, outlines of median saggital sections were drawn with a camera lucida at an enlargement of 58 diameters. These drawings were then traced with a planimeter to get the areas in sq. mm. The figure given in the table is the mean size for several glands.

proportion to body weight than in controls. From this observation it seems clear that the intestinal growth is not dependent upon the gonadotropic hormone as is the gonad and indirectly the comb. Riddle and his co-workers have shown that prolactin induces an overgrowth of liver and intestine. Is it possible that prolactin is the cause of splanchnomegaly in the White Leghorn limited diet birds? If prolactin is secreted by the acidophiles, as suggested by Riddle and Schooley, we have in the White Leghorn limited diet chicks the cellular basis for an explanation of splanchnomegaly. While the basophiles are suppressed or retarded, the acidophiles are as prominent or more so than in controls.

If this interpretation is correct, then we should also find splanchnomegaly in estrone-injected chicks where the acidophiles are well developed and the basophiles retarded. Breneman (unpublished data) has compared estrone-injected chicks with controls. He finds that in the experimental birds intestinal weight and length and liver weight are higher in proportion to body weight by 30 to 90 per cent.

In castrates we find the reverse condition; that is, the basophiles are accelerated in their development while the acidophiles are retarded. There should be no splanchnomegaly in castrates and Breneman's preliminary data indicate that intestinal size and liver weight are less in proportion to body weight than in controls.

Limited diet restricts the development of the pituitary, affecting particularly the basophiles. Castration speeds up the development of the basophiles and causes degranulation and regression of the acidophiles. Pituitaries of thirty-day castrates fed on a limited diet were intermediate in size (5610) between pituitaries from castrates (7967.5) and those from limited diet chicks (4288) of the same age (Table III). The two types of chromophilic cells are also intermediate in their development, the basophiles being more highly developed than in limited diet birds and less well developed than in castrates. The acidophiles are better developed than in castrates and less so than in limited diet birds. The 15-day limited diet castrates were very similar to 15-day limited diet chicks.

Castrate Pituitaries

That the removal of the sex glands has marked effects upon the pituitary is well known. While there are conflicting statements in the literature, Severinghaus (1937) says in his review, "It seems there is an increasing unanimity in the belief that the major change in the castrate pituitary is an increase in the basophiles." He further says that most authors have reported a reduction in size and degranulation of the acidophiles. Schooley (1937), working with castrate pigeons, described

an increase in the size of the pituitary. At 1.3 months after castration, normal and castrate pituitaries weighed the same. At 1.7 months castrate pituitaries were 50 per cent heavier than pituitaries from controls. The basophiles had increased in number and size. By 2.5 months the acidophiles were few and atropic.

To date my studies of pituitaries of castrate chicks have been limited for the most part to birds from 10 to 56 days old. A few 90-day capons reared under adverse conditions were examined and a few Buff Leghorns weighing about four pounds each and probably about seven or eight months old were checked especially for signet-ring cells and aging effects. Most castrations were performed between 36 hours and 6 days after hatching—a few at 14 days.

At 10 days, even though castrated at 36 hours, there were few, if any, significant changes in the pituitary. It is possible, of course, that a study of additional material might show some effects at 10 days, for at 12 days, basophilic cells were definitely increasing in size and at 15 days the changes were very evident. At 20 days, many more basophilic cells were affected and the nucleoli of such cells had become enlarged. The basophiles in the mid-juxta neural region were first affected. The effect extended next into the posterior end and finally into the anterior. In all later stages studied including 25, 30, 35 and 56-day-old castrates, the basophiles continued to increase not only in number but in size as well (see Fig. 2, Plate IV for comparative sizes of these cells at different ages). The nucleoli also continued to enlarge during this period. In extreme cases it (sometimes two or three) occupied most of the nuclear space. In fact the enlarged nucleoli give the best landmark of the castrate pituitary (Fig. 14, Plate II). The Golgi material appeared much the same as in actively secreting basophiles of controls.

The mitochondria, on the other hand, were larger and more numerous, especially in the large basophiles of older castrates (Fig. 13, Plate II). Not only do the mitochondria increase in number and size, but they may become vesicular and fuse as in old fowls. This observation leads one to question whether aging effects may not appear earlier in castrate pituitaries than in non-castrate pituitaries and to suggest that such changes might account for the results of Bates, Lahr and Riddle (1935), who report that the pituitary of the steer showed a low gonadotropic potency when compared to pituitaries from embryos, calves, adult bulls, normal cows and cows in early and late pregnancy.

A second major change in castrate pituitaries, perhaps equally marked and equally significant as that which occurs in the basophiles, is the regression of the acidophiles. In controls these cells are very numerous in young chicks at the time of castration. Following castration de-

TABLE III

Average weights[10] *of body, comb and gonads of controls and experimental White Leghorn male chicks*

Treatment		Age				
		10-day	15-day	20-day	25-day	30-day
Controls	No. used	32	18	25	29	30
	body	76.8	106	172.8	190.7	272.3
	comb	34.8	115.4	229.48	316.6	1336.9
	gonad	19.5	36.9	52.36	61.02	86.05
+5 I.U. estrone	No. used		4			16
	body		107.5			245.4
	comb		43.6			947.1
	gonad		33.7			75.5
	days injected		4–14			2–39
+100 I.U. estrone	No. used			14		10
	body			125.5		214.2
	comb			50.3		620.
	gonad			20.7		51.25
	days injected			3–19		3–29
+500 I.U. estrone	No. used		4	14		9
	body		99.5	119.9		221.7
	comb		26.5	39.6		251.7
	gonad		16.2	14.7		49.2
	days injected		4–14	3–19		3–29
+0.01 mg. O.B[9]	No. used					10
	body					239.1
	comb					1412.5
	gonad					72.1
	days injected					3–29
+0.0005 mg. Stilbestrol	No. used					12
	body					236.3
	comb					779.6
	gonad					54.5
	days injected					3–29
+0.2 mg. Stilbestrol	No. used					12
	body					235.6
	comb					123.7
	gonad					31.2
	days injected					3–29
Limited diet +0.01 mg. T.P.[8] Third day +1.5 mg. testosterone in pellet form	No. used		12			
	body		50.7			
	comb		324.2			
	gonad		6.8			
	days injected		4–14			

TABLE III—*Continued*

Treatment		Age				
		10-day	15-day	20-day	25-day	30-day
Limited diet +0.07 mg. O.B	No. used		7			
	body		63.4			
	comb		198.5			
	gonad		15.3			
	days injected		3–14			
Castrates	No. used				12	9
	body				230.1	286.7
	comb				123.1	266.4
Castrates +5 I.U. estrone	No. used					7
	body					243.
	comb					217.4
	days injected					
Castrates +0.05 mg. O.B	No. used					10
	body					251.6
	comb					1374.8
	days injected					10–29
Limited diet	No. used		31	12	30	27
	body		61.2	74.1	95.5	158.9
	comb		23.9	24.9	42.1	235.1
	gonad		15.9	15.3	17.7	51.0
Limited diet castrates	No. used		7			5
	body		57.1			124.2
	comb		17.6			62.3
Limited diet castrates +0.01 mg. O.B	No. used		7			
	body		56.2			
	comb		23.7			
	days injected		5–14			
Limited diet +500 I.U. estrone	No. used			14		
	body			76.7		
	comb			20.0		
	gonad			9.0		
	days injected			3–19		
+2 I.U. Prolactin	No. used			14		
	body			132.8		
	comb			120.0		
	gonad			34.9		
	days injected			3–19		

[8] Testosterone-propionate.

[9] Oreton B (dihydroandrosterone-benzoate).

[10] Body weight in grams; comb and gonad weights in milligrams.

granulation takes place and then the cells gradually regress. In some castrate pituitaries only a few granular acidophiles persist, and as many of the chromophobes have changed into basophiles, the gland is composed largely of basophiles.

At all stages studied castrate pituitaries were larger than the pituitaries of controls (Table II) and as the acidophiles had regressed and the chromophobes had not multiplied, the enlargement was due to the increase in number and growth of the basophiles.

No signet-ring cells have been observed in any castrate chicks, even in the Buff Leghorns seven or eight months old.

While only a few pituitaries from gonadectomized females have been studied, it is clear that changes are similar in both sexes.

There is considerable variability with respect to the changes in castrate pituitaries. This is true even though the chicks are fed the same food, kept in the same room and the same cages. Those carried through at the same time, however, are more nearly alike. Adverse conditions retard the changes in both basophiles and acidophiles. One lot of castrates was kept in the country for 90 days. They were not well cared for and disease killed a number of the birds. Pituitaries from these capons showed fewer modified basophiles and the acidophiles had regressed much less than in younger castrates reared under better conditions.

Another lot of castrates was fed on a limited diet (limited in quantity) for 30 days. The size of the pituitaries from these chicks was even less than in 30-day controls and castration changes in both basophiles and acidophiles were retarded. For relative sizes of pituitaries, see Table II.

Injections of Androgens and Estrogens

Because of the many conflicting observations and interpretations of the pituitary gonad relationships, Breneman, in a series of papers, partly published and partly unpublished, has sought to get further data on the problems by injecting varying amounts of both androgens and estrogens into normal or untreated chicks, castrates, and limited diet chicks. I shall describe the cytological changes in the pituitaries following injections and attempt a correlation of these observations with the experimental facts.

As previously described, functional basophiles are present in chicks 10 days after hatching. Acidophiles are well developed and filled with granules at the time of hatching. The size and number of these acidophiles increase as the gland enlarges. The basophiles also gradually increase in number as growth progresses, but since there were but few

such cells at 10 days, the number of basophiles increases relatively at a more rapid rate than the acidophiles.

Effects of Androgens on Normal Chicks

Two experiments were performed to test the effects of Oreton B (dihydroandrosterone-benzoate) on the pituitaries of normal birds. First, chicks were injected daily with 0.01 mg. Oreton B from the third to the twenty-ninth days after hatching and killed on the thirty-first day. Second, chicks were injected daily with 0.125 mg. Oreton B from the fifth to the ninth and from the eleventh to the fourteenth days after hatching and killed on the twentieth day.

In the 31-day-old birds of the first experiment, many basophiles had developed to the point where they were functional, but the number was not as great as in uninjected controls. The acidophiles still stood out prominently. Most were filled with granules while some had degranulated. The pituitaries were smaller than in 30-day uninjected controls, the ratio being 4970 to 5958.

In the second experiment, the amount of Oreton B was greatly increased (0.125 mg.) but the injection period was restricted. This greater dosage did not produce effects which were very different from those of the lesser dosage. There were some functional basophiles in these 20-day chicks, but the number was relatively smaller than in uninjected controls.

As in the first experiment, the pituitaries were smaller than in controls of the same age, 4216 to 4715.

Breneman (1937) finds that the testes are smaller in androgen-injected chicks than in controls and this is the result expected since the basophiles are reduced following androgen injections.

Castrates Injected with Oreton B

The following data are far from adequate but they are indicative. Twenty-day-old chicks were castrated on the fifth day and injected daily with .125 mg. Oreton B (dihydroandrosterone-benzoate) on the fifth to ninth and the eleventh to fourteenth days. There were no indications of castration effects on the pituitaries. In fact, the glands were smaller than those of 20-day controls. According to the method adopted for measuring size, these figures are 4715 for controls and 4120 for the injected castrates.

Thirty-day-old chicks castrated on the fifth day were injected daily from the tenth to the twenty-ninth days with 0.05 mg. Oreton B. The pituitaries showed a marked increase over those of controls in the number

and size of the basophilic cells, and they also showed a degranulation and recession of the acidophiles. The pituitaries were decidedly castrate-like. This is true with respect to the size of the gland as well as the cellular picture. The relative sizes of the pituitaries were, castrates 7084, controls 5958, injected castrates 6460. The relative weights of the combs are also interesting. For the 30-day controls the average weight was 1336.9 mg., for the 30-day castrates, 266.4 mg., and for the injected birds, 1374.8 mg. Of course the comb growth of the injected birds is due to the direct effects of the injected Oreton B. This increase demonstrates, however, that the dosage is large enough to be 100 per cent effective with respect to comb growth, and hence might have been expected to have had more effect on the pituitary than was actually the case.

These two experiments, while not conclusive, demonstrate that castration effects can be modified and possibly prevented by injections of Oreton B.

Effects of Estrogens on Normal Chicks

Estrogens were injected in varying dosages and for different periods of time into normal birds. Fifteen, 20-, and 30-day age groups were used. For the 15-day group, injections were made daily from the fourth to fourteenth days; for the 20-day group from the third to the nineteenth days; and for the 30-day group from the third to the twenty-ninth days. Three dosage levels were used, 5, 100, and 500 I. U. The major effects for all dosages and for all ages were the retardation of the development of functional basophiles and the restriction of the size of the pituitaries. The extent of the effects varied with the dosage, and with one exception results are in proportion to the quantity of estrone injected, the greater the quantity, the more pronounced the effects. Too much stress, however, should not be laid upon this exception for the size given is an average for only three pituitaries. Considering the variations to be found, it is remarkable that results are as consistent as they are. At 15 days pituitary size for controls was 3378.5; for chicks injected with 5 I. U. estrone, 2740; and for chicks injected with 500 I. U. estrone, 2415. At 20 days pituitary size for controls was 4715; for chicks injected with 100 I. U. estrone, 3223; and for chicks injected with 500 I. U. estrone, 3696.6. At 30 days pituitary size for controls was 5958.3; for chicks injected with 5 I. U. estrone, 4362.5; and for chicks injected with 500 I. U. estrone, 3926.6 (Table II).

As previously stated, functional basophiles are present in 10-day controls and the number increases with age. In 15-day chicks injected with 5 I. U. estrone, functional basophiles are present but when compared with controls the number is reduced; when injected with 500 I. U. estrone

no functional basophiles are present. These observations are not only consistent with respect to comparative pituitary sizes but also with respect to comb and gonad weights (Table III). In 10-day controls the average comb weight was 34.8 mg. and the gonad weight 19.5 mg. At 15 days these weights had increased to 115.4 and 36.9 respectively. The weights of comb and gonad in 15-day chicks injected with 5 I. U. estrone were 43.6 and 33.7. The gonad weights in these two 15-day-old groups are similar, 36.9 and 33.7. Comb weights, on the other hand, are very different, 115.4 for controls as against 43.6 for the injected birds. As comb growth is due largely to the effects of androgens and as the secretion of male hormone by the testes is dependent upon the action of gonadotropic hormone secreted by the basophiles of the pituitary, comb growth is indirectly a measure of basophilic activity as well as a measure of male hormone output. On the basis of comb growth, then, the basophiles should have been inactive in the 15-day estrone-injected birds, and by direct observation they were inactive.

At 20 days the comb in controls has increased in size to 229.48 mg. and the gonad to 52.36 mg. These weights in 20-day birds injected with 100 I. U. estrone were 50.3 mg. and 20.7 mg. When injected with 500 I. U. estrone they were 39.6 mg. and 14.7 mg. In both these experimental groups the basophiles were undeveloped and the small sizes of the combs and gonads indicate clearly that little or no gonadotropic hormone could have been released. It should be noted that the 500-unit dosage produces a greater inhibitive action than the 100-unit dosage, a fact which makes me think that the greater size recorded for the pituitaries of the 500-unit dosage is due to variation in gland size. If greater numbers of pituitaries had been measured, this inconsistency probably would not have occurred.

The combs in 30-day controls averaged 1336.9 mg. and the testes 86.05 mg. These weights are marked increases over 20-day controls and they demonstrate the high potency of endogenous gonadotropic and male hormones at this time. In 30-day chicks injected with 5 I. U. estrone, comb and gonad weights were 947.1 and 75.5; when injected with 100 I. U. estrone, these weights were 620.0 [2] and 51.25,[2] and when injected with 500 I. U., they were 251.7 and 49.2. In all three experiments there is a reduction in size of both comb and testis. The testicular weights, however, with the 100 and 500 unit dosages, were essentially the same. The pituitary sizes for the controls, 5- and 500-unit injection groups, were 5958.3, 4362.5 and 3926.6 respectively. The basophilic picture is in accord with these facts. There are fewer large basophiles in the 5-unit

[2] These weights were for 31 days instead of 30 and hence the figures are slightly higher than they should be.

estrone-injected chicks than in controls, and there are still fewer large basophiles in the 100- and 500-unit injected groups.

Breneman has performed two experiments with daily injections of Stilbestrol from the third to the twenty-ninth days. Two dosage levels were used, 0.0005 mg. and 0.2 mg. The effects were similar to those obtained when 100 and 500 I. U. of estrone were injected. See Table II for comparative pituitary sizes and Table III for weights of combs and testes.

Limited Diet Chicks Injected with Estrone

Two lots of limited diet chicks were injected with estrone daily from the third to the nineteenth days and killed on the twentieth day. One lot was given daily injections of 100 I. U. and the second 500 I. U. Since estrone retards the development of the basophiles and since a limited diet does likewise, we have two forces acting in the same direction. The combined effects are greater than with either factor acting alone. The acidophiles still stand out prominently, however. The pituitaries of the limited diet estrone-injected chicks are smaller than in 20-day limited diet chicks or in 20-day normal diet chicks injected with 100 I. U. estrone (Table II).

Castrates Injected with Estrone

Twenty-day-old chicks castrated on the fifth day and injected daily with 100 I. U. estrone on the fifth to the ninth and the eleventh to the fourteenth days, showed no effects of castration on the pituitary. Some functional basophiles were present, but no more than were found in non-castrate uninjected controls. The size of the pituitaries of the experimental birds was 4843, approximately the same as in the controls which was 4715.

Pituitaries from 30-day-old chicks castrated on the fifth day and injected daily with 17.5 I. U. estrone from the tenth to the twenty-ninth days were decidedly castrate-like. The basophiles were much enlarged as were the nucleoli, while many of the acidophiles were partially degranulated and reduced in size. The basophiles showed no more signs of degranulation than in uninjected castrates. Degranulation of the acidophiles was no doubt primarily a castration effect and not due to the estrone injections.

The generally accepted interpretation of estrogen effects on the pituitary is that the estrogens inhibit the secretory activity of the anterior lobe and my observations favor this point of view. Severinghaus, however, says it is not possible to interpret the cytological data in this way if by secretory activity is meant the production and release of secretory substances. He believes there is a marked degranulation of both baso-

philes and acidophiles in estrogen-treated rats—also hypertrophy of the mitochondria. Estrogen then brings about a release of secretory materials. He is not so certain about the effects of estrogens upon the production of secretory substances. He argues, however, in favor of a stimulating effect upon production.

In the chick, as far as studied, the basophiles are restricted in their development and the acidophiles are not degranulated following estrogen injections. Instead of an enlargement of the entire anterior lobe, as Severinghaus and others report in the rat, there is actually a decrease in pituitary size in the chick. This decrease holds for dosages as low as five units daily for 26 days and for estrone injections into castrates, limited diet chicks, and controls. These size relations are consistent with the point of view that basophilic development is restricted and that acidophilic development is but little modified.

Brooding Hens

Brooding hens of known ages have been difficult to find, but Dr. Martin of Purdue University supplied two Barred Rocks, one of which was about 18 months and the other 30 months old. A larger sampling would have been better, but certain facts stand out clearly. In laying Barred Rock hens and in 25-day chicks of the same breed, the functional basophiles are very similar in size, shape, distribution and staining reactions to those found in White Leghorns. The cytoplasm stains a light green; the nucleus is large and stains similarly to the cytoplasm; and there are one or two spherical nucleoli. In the brooding hens the large basophiles have been reduced in number and have been replaced by much smaller cells, the nucleus of which is pycnotic and stains uniformly throughout except for the slightly darker nucleoli. In a comparatively small number of these cells, with pycnotic nuclei, the cytoplasm is also pycnotic. Such cells are inactive basophiles reduced in size until they are not much larger than chromophobes. With the exception of the extreme posterior end, they are distributed throughout the gland, but are most numerous in the mid-region.

There is a possibility that the acidophiles in the anterior end (A2) are more numerous than at any other time during which they have been observed, but the evidence is too meagre for any definite statements. The acidophiles in the posterior end (A1), however, are no more numerous than in laying hens.

The Pituitary in Old Age

In discussing changes described as aging, it should be remembered that aging effects are relative and variable. When do these effects begin?

Minot has shown that the growth rate of the rabbit embryo is high in early stages, and then rapidly diminishes. Is this an aging effect? Robbins and others (1928), in their book on growth, give data with respect to egg production in the domestic fowl. Hens one year old lay the largest number of eggs and thereafter the number gradually grows less. Does aging in the hen begin at two years or even earlier in the pituitary and sex glands? Ordinarily, in discussing old age, we do not include these early changes; nevertheless they cannot be ignored and no one can say just when old age begins.

With our increasing knowledge of the endocrine glands and the part they play in physiological processes, the question has arisen whether changes in the activities of these glands may not be primary causes of aging. This question applies particularly to the pituitary, which is the source of several hormones and which influences directly or indirectly other endocrine glands and many metabolic activities.

Unfortunately, our information about pituitaries of aging animals is rather limited and perhaps some of it is erroneous. Carlson (Cowdry, 1939) quotes Parsons and Cooper to the effect that neutrophiles (chromophobes) increase throughout life and that basophiles invade the intermediate and posterior lobes. Carlson also states, "This gland appears, anatomically and cytologically, remarkably stable, from the age of 20 on to 80." He concludes that there is no valid evidence for either hypo- or hyper-function of the pituitary in old age in man. Severinghaus (1937) finds an increase in the number of basophiles in women over sixty years of age and accounts for this change by assuming old females to be physiological castrates. This suggestion, however, would not account for changes in the male nor are we certain that there is complete cessation of sex hormone secretion after the menopause. In fact, menopause urine is high in FSH, but there may be some source other than the pituitary. Hellbaum (1935) studied the gonad-stimulating activity of pituitaries from seven old mares which were beyond the reproductive period. Five of these pituitaries induced greater responses, judging by ovarian weight, than did pituitaries from pregnant mares, non-pregnant mares, stallions, geldings, colts, and foetuses. Two of these mares, however, which appeared older than the others, induced only slight follicular enlargements, results similar to those obtained with pituitaries from geldings more than seventeen years of age.

Hellbaum reports in a footnote in this same paper that in human beings of extreme old age, who have been afflicted with disease, the FSH potency of the pituitary was decidedly low. Whether this condition was due to disease, old age, or a combination of the two factors, he did not discuss, but inferred that it was due to old age plus disease.

Schooley (1937) states that both acidophiles and basophiles cease to function in senile animals.

My own observations on the pituitaries of old chicks have been limited because of the lack of availability of old birds of known age. Fortunately, however, I was able to get two White Leghorn hens thirteen years old from Professor T. B. Clark of the Department of Animal Husbandry of the University of West Virginia and five cocks, varying in ages from five to nine years from Dr. H. D. Goodale of Mount Hope Farm. Three of these cocks were Inbred Leghorns and two were Rhode Island Reds. Two of the leghorns were six and one eight years old. One Rhode Island Red was nine years old, the other five. A fourth White Leghorn cock, six years old and originally from the Mount Hope Farm, was obtained from Professor Martin of Purdue University. Professor Martin also furnished me with White Leghorn hens one, two, and three years old. Additional old fowls of unknown ages were obtained from the local poultry house.

While there is some variability, as expected, the pituitary changes in old fowls are consistent. The changes are gradual and all glands and all cells are not affected at the same time nor to the same degree.

In young birds the mitochondria in the basophiles are granules, varying in size and usually distributed at random through the cytoplasm. With fuchsin they stain uniformly a purplish red. The number is variable and seems to be greatest in older castrates. The granules may become vesicular and in older birds the vesicles may fuse to form larger vesicles, some of which are irregular in outline while others are spherical (Figs. 15 and 17, Plate II). There may be several of these larger fusion masses or only one.

With the acid fuchsin method of staining, the central areas of the masses are golden yellow while the peripheries are dark red. In osmicated material the peripheral region becomes black, while the central area looks much as it does in stained preparations. These mitochondrial spheres continue to enlarge. They not only may fill the cell (Fig. 11, Plate II), but the cell may eventually break down, leaving the spheres to occupy intercellular spaces. Since many basophiles may be affected, the golden masses present a striking picture in contrast to the background of the methyl-green stain. Figure 5, Plate I is an outline sketch which shows how numerous these spheres may be.

In speaking of the large spheres as vesicular mitochondria, I do not mean to imply that they are unmodified mitochondria. Just what mitochondria are from a chemical point of view I do not know, but they are probably combinations of several chemical substances includings proteins

and lipids. Perhaps the principal change is due to the gradual infiltration of lipoidal substances.

Not all basophiles, however, become modified as described. Others, without undergoing such modifications, shrink in size and become pycnotic. In very old birds such as the two 13-year-old White Leghorn hens and the 9-year-old Rhode Island Red cock, it is doubtful whether any of the basophiles could have been functional. The entire glands were degenerate structures. In the 5-year-old Rhode Island Red and the 6-year-old Leghorns, a number of basophiles were certainly still functional but in the 8-year-old Leghorn a smaller number were functional.

While I have for the moment concluded that the starting point of the large spheres is a fusion of the vesicular mitochondria, I wish to point out that the tiny vesicles might possibly arise de novo independently of the mitochondria and that if they did, it would be practically impossible to distinguish between the two if they both reacted to the stain in the same way. It should be remembered, of course, that vesicular mitochondria are common in many animals and that their fusion is not uncommon. The most notable example of fusion is found in the formation of the Nebenkern of spermatids.

The formation of vesicular mitochondria and their fusion is also found in capons. There the evidence is even more convincing that vesicular mitochondria do actually fuse.

The acidophiles are very much reduced in number in all old birds, but they never completely disappear. Whether they are still functional cannot be stated from the cytological evidence alone. The cells, however, are much smaller than the large functional acidophiles of younger birds and the granules stain a darker purplish red.

In some of the acidophiles (A1) of all old fowls studied a spherical vesicle is found in the cytoplasm. See Fig. 17, Plate III for size and position. It does not stain but has a slightly yellowish or golden tinge. Whatever the substance is, it is not dissolved by the cytological reagents used. At first I thought it might be formed by a fusion of the mitochondrial granules much as found in the basophiles, but the vesicle and mitochondria are present at the same time in the same cell. On the other hand, the vesicle may arise by some modification of the Golgi substance but there is no direct evidence for such an origin. The Golgi substance in osmicated material is similar to that found in acidophiles of younger fowls. Origin de novo is a third possibility. The percentage of cells having this body was not determined, but as age progresses, the number increases.

A vesicle similar to that observed in the fowl is also found in the acidophiles of an old guinea-hen. Here the body is larger in comparison

to cell size, more conspicuous, and it seems there is one in every acidophile.

One of the first indications of age in the pituitary of fowls is the formation of acini (Fig. 6, Plate I), first at the posterior end of the gland, then near the periphery of the ventral surface, and finally at the anterior end. The juxta-neural and central regions are least affected. These acini are filled with material, perhaps colloidal in nature, but there is no direct evidence that it is secreted by the limiting cells. In fact, the cells are not granular and do not have the appearance of either actively secreting basophiles or acidophiles. Acini first appear in White Leghorn hens between two and three years old.

Another change common to pituitaries of old fowls is the presence of pigment granules. In extremely old birds most cells which have not already become highly degenerate, particularly those at the anterior end, are affected and the cytoplasm becomes filled with them. The granules are present in White Leghorn hens as early as two years of age.

Finally the chromophobes become reduced until only a few remain. The entire gland from a cellular point of view is a highly degenerate structure. It cannot be functional. All changes enumerated are gradual and it is only in very old birds that complete degeneration is found.

Gonads of the six old cocks were studied as well as pituitaries. The two 6-year-old Leghorns had large testes and sperm formation was actively taking place in all cords. In the third 6-year-old White Leghorn one testis was only half the size of the other and sperm formation was reduced in each. Both testes of the 8-year-old Inbred Leghorn were only about half size and sperm formation was very much reduced. Functional basophiles were present in all four pituitaries but the number was much less in the pituitary from the 8-year-old bird. Sperm formation in the 5-year-old Rhode Island Red was more reduced than in the 6-year-old Leghorns, in fact, even more so than in the 8-year-old Leghorn. In the 9-year-old Rhode Island Red, there were no indications of sperm formation and the cords had largely disappeared. A mass of cells with remnants of cord walls was all that remained. The pituitary of this bird probably had no functional basophiles. It seems clear that aging had progressed more rapidly in the Rhode Island Reds than in the leghorns—a varietal difference in all probability. The close correlation between the development of functional basophiles on the one hand and sperm formation and degeneration of the spermatic cords on the other is no mere coincidence. Changes within the testes are evidently dependent directly upon the activity or lack of activity of the secretory cells of the pituitary, and of the cells the basophiles probably play the more important rôle.

Pituitaries from White Leghorn hens one, two and three years of age were also studied. Acini and pigment granules were present at two and one-half and three and one-half years, but not at one year.

By way of summary it may be stated that pituitary changes in aging fowls are as follows:

1. Formation of acini, beginning as early as two and one-half years in White Leghorn hens.
2. Fusion of mitochondria and hypertrophy of the mitochondrial material in many of the basophiles.
3. Absence in old age of all functional basophiles. This is true for the two hens 13 years old and probably true for the 9-year-old Rhode Island Red cock.
4. Only a few granular acidophiles are present in all old hens and it is doubtful whether they can be functional. The same is true for old cocks. Younger cocks have not been studied.
5. Pigment granules are abundant in pituitaries of some old birds; less so in others.
6. Gradual disappearance of all, or practically all chromophobes.
7. Near the end comes degeneration of all cells. In cells on the way toward degeneration nuclei and cytoplasm are pycnotic.

These observations are at variance with the conclusions of Cooper and Parsons that the neutrophiles increase in number in old age, and with Carlson's conclusion that the pituitary in man from ages twenty to eighty years remains stable. Neither do these observations support Severinghaus' conclusion that the basophiles increase with age. They may increase in the fowl up to a certain point but after that, degenerative changes set in. Very definitely the evidence in old fowls points to pituitary changes in which all activity gradually diminishes and finally ceases. The gonad changes, in so far as sperm formation and cord structure are concerned, parallel closely the pituitary changes. Similar parallel changes must also occur in the ovaries of hens where egg production gradually grows less after the first year.

The data presented, however, give no conclusive evidence whether the pituitary changes are primary causal factors of aging processes in other parts of the body or whether they merely parallel other changes.

Conclusions

1. Cytological observations are in conformity with experimental data.

2. Functional basophiles are present in both male and female White Leghorn chicks at ten days of age. In Barred Rocks and Rhode Island Reds these cells do not function until after twenty days of age.

3. Acidophiles and basophiles arise from chromophobes and may regress, but whether they return to undifferentiated chromophobes from which either type of chromophile may arise is still questionable.

4. The evidence indicates there are two kinds of acidophiles.

5. Basophiles and acidophiles cannot be differentiated in early transitional stages on the appearance of the Golgi substance.

6. There is no division of the anterior pituitary in early embryonic development into an anterior and posterior lobe.

7. Limited diet as well as unfavorable conditions in general restricts the development of basophiles but has no adverse effects upon the acidophiles.

8. Castrate pituitaries are characterized by an unusual growth of the basophiles and a regression of the acidophiles.

9. Changes in the pituitary brought about by castration may be retarded by injections of estrogens and androgens.

10. In normal untreated chicks both estrogens and androgens retard the development of basophiles but have little or no effect upon the acidophiles.

11. When estrogens are injected into limited diet chicks, basophilic development seems completely blocked.

12. Acidophiles remain well developed in limited diet and estrone-injected chicks. Under both these conditions the intestine and liver are enlarged. These facts lend support to the suggestion that the acidophiles secrete prolactin or a growth-promoting substance affecting the intestine and liver.

13. In brooding Barred Rock hens many of the basophiles are much reduced in size and the nuclei are pycnotic. The A2 cells may be in a state of increased activity.

14. Aging effects begin between two and three years in the pituitaries of White Leghorn hens. The effects are progressive and by thirteen years the gland is probably not functional. In the nine-year-old Rhode Island Red cock the pituitary was not functional and sperm formation had ceased entirely.

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THE VERTICAL MIGRATION OF THE COPEPOD ACARTIA TONSA UNDER CONTROLLED ILLUMINATION

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Introduction

Vast numbers of plankton organisms are known to rise toward the surface of the water at night and sink downward during the day. This vertical migration was first studied by the great oceanic expeditions of the last century, particularly that of the " Challenger " in 1872 (Murray and Hjort, 1912). The first laboratory investigations were those of Groom and Loeb (1890) and Parker (1902). The earlier work has been extensively reviewed by Russell (1927) and Kikuchi (1930).

It was soon apparent that light was the main environmental factor involved, but many diverse conclusions were made as to its mode of action. These may be summarized as follows:

(1) *Phototropism overcoming geotropism.*—Parker (1902) found that *Labidocera aestiva* rose at night because of its negative geotropism, but sank during the day because this was overcome by its negative phototropism.

(2) *Light changing the sign of geotropism.*—*Cyclops albidus* was found to be geopositive in the light and geonegative in the dark (Esterly, 1907). Field observations on *Cyclops* by Worthington (1931) and Southern and Gardiner (1932) support this view.

(3) *Optimum light intensity.*—Rose (1925) placed various copepods in a horizontal tube with a graded light intensity, and found that the animals gathered in an intermediate zone. Field observations indicate that *Calanus* (Russell, 1926, 1928, 1934), *Metridia* (Clarke, 1933, 1934) and other forms follow a zone of optimum intensity.

(4) *Relative optimum intensity.*—*Daphnia* was found by Clarke (1930) to have a " primary " negative phototropism, which is strengthened by an increase of light intensity. When the intensity is reduced, the animal acquires a " secondary " positive phototropism and moves toward the light as long as it is being reduced. But if the intensity is held constant, the primary sign returns, and the animal moves away from the light. There is only a relative optimum here, the animal becoming adapted to the intensity existing at the moment. Such a mechanism is suggested by field observations of *Acartia clausi* (Johnson, 1938).

Other experiments have led to diverse conclusions. Johnson and Raymont (1939) found that *Centropages typicus* moved towards the light when the intensity is either increased or decreased. Downward movements during the night, when light intensity is constant, have been reported by several field workers (e.g., Waterman et al., 1939). These authors suggest that light merely keeps in phase internal physiological cycles which regulate the migration of the organisms. Esterly (1917*b*) found that *Acartia tonsa* kept in constant darkness moved upward on two successive evenings, suggesting the influence of an internal rhythm.

In none of these studies has vertical migration actually been reproduced. Groom and Loeb (1890) watched *Balanus* nauplii performing their diurnal migrations in a glass of water on the window, while Parker (1902) noted the migration of *Labidocera* in a floating jar outside the laboratory. But aside from these early observations, there are no reports of diurnal migrations being performed under controlled conditions. Many of the conflicting results may have been caused by subjecting the animals to laboratory situations which would never be encountered in nature. Thus Loeb (1908) concluded that vertical migration is caused by the difference in temperature between warm waters near the surface and cool deep waters. This obviously cannot apply to migration in those lakes where no temperature gradient occurs (Worthington, 1931). The observation of Clarke (1930) that the phototropic sign of *Daphnia* is reversed by changing light intensity, cannot apply to the downward migration of this form beginning at midnight (Southern and Gardiner, 1932). It was therefore decided to inquire if vertical migration could be reproduced under conditions approximating natural ones as closely as is possible in the laboratory. If so, it would then be possible to alter various factors in order to find the controlling ones.

Apparatus and Procedure

Animals collected by towing an open net were brought into the laboratory and placed in tall glass cylinders exposed to window light. The cylinders were placed in a large tank filled with water to permit control of the temperature. Observations were made by removing the cylinders from the tank and noting the vertical distribution of the animals.

Specimens of the copepod *Acartia tonsa* Dana were obtained by towing an open net for five minutes at a depth of 3 meters at the head of Woods Hole Harbor. The animals were collected in a small bottle towed at the end of the net; to avoid overcrowding they were transferred to a larger bottle as soon as the net was hauled aboard. The catch was

immediately brought into the laboratory and poured into finger bowls. Individual *Acartia tonsa* were identified under a dissecting microscope, picked up in a pipette, and placed 20 each in six glass cylinders, 48.5 × 4.5 cm., filled with sea water to a height of 43–44 cm. The cylinders were placed in a black-walled tank, 46 × 66 × 45 cm., open at the top, and placed in front of a window. The tank was filled to the water line in the cylinders with fresh water, which was kept at 15° by a thermostat attached to a refrigerating unit. Surface temperature in the harbor at the time of the hauls was 18–21° C.

The animals were fed with the diatom *Nitzschia closterium*, found by Clarke and Gellis (1935) to be a favorable diet for copepods. The diatoms were cultured by the method of Ketchum and Redfield (1938). A few drops of this culture were added to each cylinder every morning. Varying the time of feeding did not affect the behavior of the copepods.

Observations were made by counting the number of animals swimming in each vertical third of each cylinder. Since it was not possible to see animals lying on the bottoms of the cylinders, they were computed by subtracting the total number of animals swimming at any count from the highest total obtained in subsequent counts in that cylinder during the day. Thus, if six animals were observed swimming in cylinder II at 8:00 A.M., while ten animals were found swimming in the highest subsequent count of the day, it was assumed that four animals were resting on the bottom at 8:00 A.M. Dead animals are thereby eliminated, since they appear in no subsequent count of animals swimming. From this data, the percentage of animals in the top third of the cylinders was computed for each observation.

The cylinder to be counted was taken out of the tank and placed on a nearby shelf. Observations in the darkroom were made by turning on a 25-watt red lamp for one to two minutes while the animals were counted. This brief exposure to light did not affect their relative position. The remaining cylinders were covered during this time. The chief source of error in this procedure lies in the necessity of removing the cylinders from the tank for counting. Any such error is probably constant under the same light conditions, but may affect the results when different conditions are being compared.

Observations

Normal Behavior

The cylinders were first placed in front of an unobstructed south window, so arranged that they were never exposed to direct sunlight. Photometric observations showed that the light reaching the tank was

of approximately the same intensity as that at a depth of 10–20 meters in Woods Hole Harbor (Oster and Clarke, 1935). Normal vertical migration was carried on under these conditions for at least eight successive days. The animals moved up rapidly immediately following sunset, and then more slowly. The downward movement commenced at midnight, and proceeded slowly until dawn, when it became more rapid. The daytime level remained relatively constant (Fig. 1). The only field observations of the diurnal movements of *A. tonsa* are those made at La Jolla, California, by Esterly (1928). When the two curves

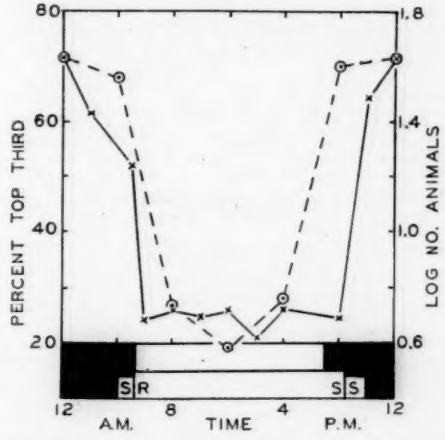

FIG. 1. Vertical distribution of *Acartia tonsa* in laboratory (solid line). Ordinate, percentage of animals in top third of cylinders; abscissa, time of day. Distribution in field (dotted line) from Esterly, 1928. Ordinate, mean logarithm of number of animals per haul; abscissa, time of day. *SR* represents sunrise, *SS* sunset. Upper bar for La Jolla, lower for Woods Hole. The shorter day in California seems to be correlated with a shorter downward movement.

are superimposed, the similarity of their shape indicates that the laboratory behavior is representative of that in the field.

An attempt was made to reproduce this behavior under more carefully controlled conditions. Room light was excluded by placing a tarpaper shelter over the tank. The cylinders were illuminated with daylight fluorescent lamps, chosen because their spectral energy distribution is approximately the same as that of daylight (General Electric Co., 1939). An opal glass diffusion screen was placed over these lamps to imitate the broad source area of window light. Four 24″ lamps were used, covered with a 14×24″ opal screen. To reproduce the

angle at which window light entered, the lamps were placed 60 cm. above the tank and 80 cm. to the side. The light now entered the cylinders at an average angle of 36° from the horizontal. Normal vertical migration continues under these conditions. The animals stay at the bottom of the cylinders during the day when the lights are on, and rise to the top at night when the lights are off (Table I).

TABLE I

Fluorescent light. Average of 60 A. tonsa at 15° C. for 48 hours; July 28–30, 1941. Observations in darkness are italicized.

Time	Percentage of Animals in Top Third of Cylinders	Time	Percentage of Animals in Top Third of Cylinders
6 A.M.	*52.2*	6 P.M.	9.55
8	13.4	8	7.97
10	3.50	10	*62.2*
2 P.M.	9.45	12 Midnight	*52.1*

Diurnal Rhythms

If this vertical migration is controlled by an internal rhythm, the normal movements should continue in constant darkness. Although the animals began their normal descent on the first morning of darkness, they did not leave the upper portion of the cylinders (Fig. 2). While no migration appears under these conditions, the normal behavior may be controlled by the coincidence of light and an internal rhythm set for the normal alteration of night and day. That this is not so is seen by the fact that the migration can be reversed by illuminating the animals at night and keeping them in the dark by day (Table II). It is significant, however, that the upward movement during the first reversed day is appreciably less than during the second. This behavior, together

TABLE II

Reversed day and night. Average of 120 A. tonsa at 15° C.
Observations in darkness are italicized.
July 31–August 1, 1941: Normal fluorescent light.
August 1–4: Reversed day and night.

Date	Percentage of Animals in Top Third of Cylinders					
	2 A.M.	8 A.M.	10 A.M.	2 P.M.	8 P.M.	10 P.M.
July 31					10.5	*51.1*
Aug. 1		*61.6*	0.81	21.6	10.8	14.3
2		14.3	*37.1*	*44.1*	*42.5*	12.1
3	18.2	15.2	*62.6*	*51.8*	*64.3*	*51.8*
4		*33.4*				

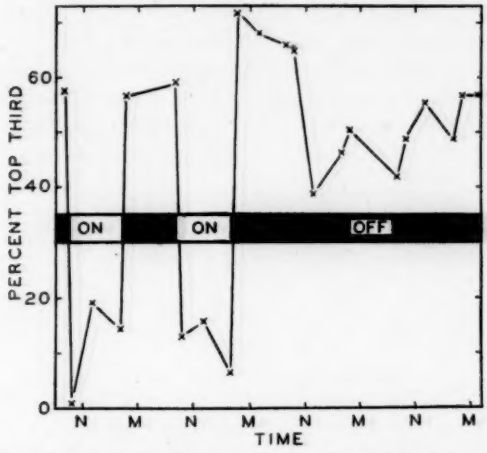

FIG. 2. Vertical distribution of *A. tonsa* in daylight fluorescent light and in constant darkness. Ordinate, percentage of animals in top third of cylinders; abscissa, time of day. N = noon; M, midnight.

with the downward movement during the first morning of constant darkness, indicates that traces of a metabolic rhythm, once set up, persist under changed conditions for one day. Although light alone seems to control the behavior of the animals under these conditions, it is possible that it is effective only if it operates in a 24-hour cycle. This is not so, however, since a 4-hour "day" can be induced (Table III).

TABLE III

Four-hour day. Average of 120 A. tonsa at 15° C.; August 4, 1941—fluorescent light. Observations in darkness are italicized.

Time	Percentage of Animals in Top Third of Cylinders	Time	Percentage of Animals in Top Third of Cylinders
10 A.M.	0.40	1:00 P.M.	*40.0*
11	*28.0*	1:15	0.40
12 Noon	*48.0*		

Changes in Illumination

The lighting conditions were now altered in order to find the specific factors controlling the migration of *A. tonsa*. No difference in behavior appeared when the opal glass screen was removed, showing that the area of the light source was of no significance (cf. Fig. 2: the first day is without the screen, the second with it). Spectral energy distribution

was found not to be a controlling factor, since incandescent bulbs were substituted for fluorescent lamps without effect. Bulbs of 25, 60 and 300 watts were used without changing the normal behavior, showing that light intensity within this range is not a factor in migration (Fig. 4).

An entirely different situation prevailed when the lamps were moved directly above the tank. Migration was now reversed, the animals mov-

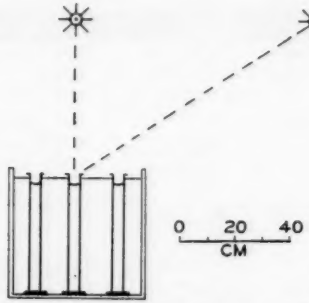

FIG. 3. Arrangement of oblique and overhead lights. Distances in centimeters.

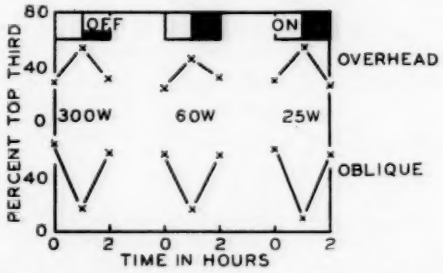

FIG. 4. Vertical distribution of *A. tonsa* in incandescent light. Top, lamps 60 cm. above tank; bottom, same, but 80 cm. to side. Ordinate, percentage of animals in top third of cylinders; abscissa, time in hours.

ing up in the light and down in the dark (Fig. 4). If the overhead lights are kept on for 24 hours, the copepods stay up throughout this period, although animals in oblique light for 24 hours stay down (Fig. 5). The animals move *up* in overhead light, or in darkness following oblique light; they move *down* in oblique light, or in darkness following overhead light. The downward movement when the overhead light is turned off lasts for only an hour. After this time the normal upward movement in darkness begins (Fig. 6). This upward movement con-

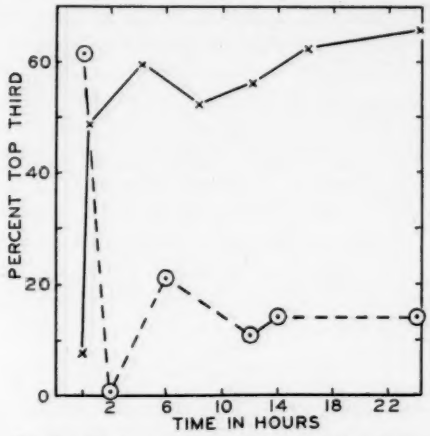

FIG. 5. Vertical distribution of *A. tonsa* in daylight fluorescent light. Solid line, lamps 60 cm. above tank; dotted line, same, but 80 cm. to side. Ordinate, percentage of animals in top third of cylinders; abscissa, time in hours.

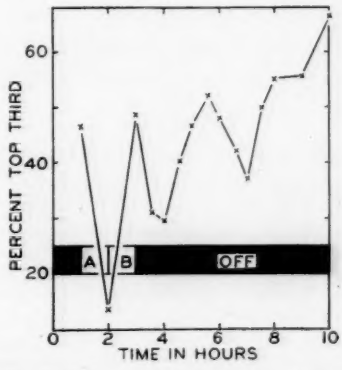

FIG. 6. Vertical distribution of *A. tonsa* under various light conditions. Ordinate, percentage of animals in top third of cylinders; abscissa, time in hours. *A*, Daylight fluorescent oblique; *B*, 25-watt overhead.

tinues for 1½ hours, to be followed by another downward movement, which in turn is followed in 1½ hours by an upward movement. If the animals have been kept up for 12 hours with overhead light, they will stay down in the dark for the next 12 hours.

A simple but dramatic presentation of these results can be made by leaving a cylinder containing *A. tonsa* in the dark for an hour. If a

weak red light is now turned on for a few moments, the animals may be seen slowly swimming about, the greater number at the top of the cylinder. If a white light, level with the center of the cylinder but several feet away, is now turned on, a remarkable change occurs in the behavior of the animals. First one and then another stops swimming and sinks slowly downward, head up, antennae outspread. Occasionally an individual swims about for a moment, but it soon stops and continues sinking downward. In ten minutes almost all the animals will be at the bottom of the cylinder. If the light is now placed on top of the tube, the animals swim rapidly upward. Some individuals, seemingly unable to get close enough to the light, hurl themselves time after time against the surface of the water.

Movements of Individuals

It has hitherto been impossible to determine whether vertical migration consists of mass movements of entire populations, or of a general trend of diverse individual movements. Thus Fig. 1 shows that about 20 per cent of the animals are on the surface during the day, and 70 per cent at night. Does this mean that only 50 per cent of the animals actually migrate, or that all the animals spend 20 per cent of their time on the surface during the day, and 70 per cent at night? To answer this question, 42 individuals were tested in oblique light, in overhead light,

TABLE IV

Individual movements of 42 A. tonsa *at 15° C.;* August 18–23, 1941.

Light Conditions	Time	Animals in Top Third of Cylinders	Average Movement of 42 Individuals*
	hours	*per cent*	
Dark	1	60.0	
	2	58.4	1.9
	3	77.8	1.8
	4	63.8	1.5
Fluorescent Oblique	5	23.3	2.0
	6	23.3	2.2
	7	27.8	1.6
	8	27.8	1.6
Dark	9	63.4	1.6
	10	66.6	1.9
	11	58.3	1.7
	12	79.2	1.2
25w Overhead	13	72.2	1.1
	14	58.3	1.2

* Each unit represents one-sixth of cylinder, or 7 to 7.5 cm.

and in the dark. The cylinders were divided into sixths numbered from top to bottom. The average distance moved per hour was measured in units representing one-sixth of a cylinder (Table IV, Fig. 7*A*). The average behavior of the entire group gives the same results as before, but the individuals are seen to be continually moving. Each animal traverses about one-third of a cylinder (14–15 cm.) every hour. Maximum movement occurs two hours after the lighting is changed. Too

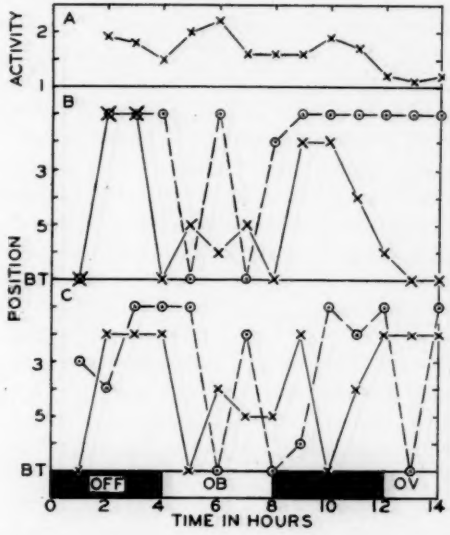

FIG. 7. *A*, average movements of 42 individuals. Ordinate, activity in units representing one-sixth of a cylinder; abscissa, time in hours. *B*, Positions of two males. *C*, Positions of 2 females. Ordinate, position of animals in sixths of cylinders, numbered from top to bottom; abscissa, time in hours; *Bt* indicates resting on bottom; *OB* indicates daylight fluorescent, oblique; *OV*, 25-watt, overhead.

few males were present to permit a statistical comparison of the sexes, but no significant differences are found between the records of individual males and females (Fig. 7). Because of the greater distances covered, the proportion of these scattered movements to the entire migration may not be as great in nature as in the laboratory. These diverse movements suggest why field observations do not show that all of the animals are right on the surface at night, and down on the bottom during the day.

Discussion

It might appear that the difference between the behavior of the animals in overhead and in oblique light is caused by the intensity of the light, since the lamp is closer to the cylinders in the overhead than in the oblique position. But measurements of light intensity show that this factor must be eliminated, since the intensities of the various sources of oblique and overhead light overlap (Table V). The only remaining difference is in the positions of the lamps.

Table V

Light intensity measured by "Photox" cell with ½" diaphragm.

Light Source	Position of Bulb: Oblique	Position of Bulb: Overhead
Window	2.8 m.a.	
Incandescent bulb		
25-watt	0.8	1.7 m.a.
60-watt	1.5	3.8
300-watt	5.2	14.0
Fluorescent lamps		
with screen	1.0	
without	2.5	

It is believed that the control of this behavior lies in the directional character of the illumination. The overhead light appeared to shine directly down the cylinders with little deflection. The oblique light, on the other hand, entering at an average angle of 36° from the horizontal, appeared to be reflected from the walls of the tank and cylinders so as to provide a more diffuse illumination (Fig. 3). Additional evidence is provided by tropism experiments in a horizontal glass trough. This trough, which could be illuminated by an incandescent bulb at either end, was surrounded by water to minimize reflection from the side. Under this highly directional illumination, *A. tonsa* always moved toward the light. By turning on first the lamp at one end and then that at the other, it was possible to keep an individual moving back and forth from end to end indefinitely. But the distribution of light in natural waters, according to recent measurements, does not show this highly concentrated character. Whitney (1941) found that the light intensity at a depth of 3 meters in Woods Hole Harbor was distributed as follows:

overhead 12 units
towards sun 16 units
horizontally 2 units
from below 1 unit

Although similar measurements could not be made in the cylinders, the oblique light appeared to provide a comparable situation. Overhead light, or the light in the horizontal trough, seemed to be of a much more concentrated character. It is believed that this difference in the concentration of the light is the only factor that can account for the difference in behavior.

This view is supported by several reports in the literature of animals which are positive to direct light, but which move down in diffuse light and up in the dark. Such behavior has been found in the larva of *Corethra plumicornis* (Harper, 1907), in *Branchipus serratus* (McGinnis, 1911), *Daphnia pulex* (Dice, 1914), and *Sagitta bipunctata* (Esterly, 1919). Two of these three relations have been described for *Cyclops albidus* (Esterly, 1907), for the nauplii of *Balanus perforatus* (Ewald, 1912), for *Paramoecium* and the larvae of the echinoid *Diadema setosum* (Fox, 1925), and for six Crustacea (Kikuchi, 1938).

In diffuse light, all parts of the photoreceptor will receive illumination of equal intensity. In direct light, on the other hand, that part of the receptor facing the source will be strongly illuminated, while much less light will come from the side. There is no need for the light to be perfectly direct or diffuse, since a threshold ratio of front light to side light may determine the behavior.

We may then conclude that:

(1) *Acartia tonsa* sinks downward in light which is not highly directional. This behavior, together with its upward movement in darkness, is believed to account for the normal vertical migration of this form.

(2) *A. tonsa* moves toward a source of highly directional light. Such illumination is not likely to be encountered in nature.

This theory will explain the paradox of an animal which moves toward the light in the laboratory, but away from it in the field. It seems to account for the fact that positively phototropic plankton do not come to the surface of the sea during the day, and also, that positively phototropic insects do not fly up to the sun.

If light alone controls the movements of these animals, we could not account for the oscillating movements in darkness following overhead light (Fig. 6). The movement toward the light, however, may result in fatigue, and the animal will then sink down. On recovery, the animal swims up, to sink down again when fatigued. Such oscillations may explain downward movements beginning at midnight, reported from both field and laboratory (Fig. 1), as well as the upward movements in constant darkness observed by Esterly (1917*b*).

There is no justification for applying the results of these experiments to vertical migration in general. Thus, while *Acartia tonsa* continues its response to light for at least 48 hours, another species of the same genus, *A. clausi*, becomes indifferent after 30 minutes (Johnson, 1938). Experiments with *Centropages hematus*, a copepod found together with *A. tonsa*, show that it performs no migration when exposed to window light. An average of 52.1 per cent of the animals remained in the top third of the cylinders throughout the day and night (Table VI). Every field observation shows that different genera and species migrate at different times and rates. Differences between broods of the same

TABLE VI

Centropages hamatus. Average of 120 animals at 15° C. for 24 hours. Window light, July 11–12, 1941.

Time	Percentage of Animals in Top Third of Cylinders	Time	Percentage of Animals in Top Third of Cylinders
2 A.M.	56.4	2 P.M.	53.2
4	56.2	4	66.7
6	39.3	6	56.1
8	44.9	8	49.0
10	48.3	10	51.8
12 Noon	59.4	12 Midnight	44.6

Average time of sunrise, 5:16 A.M.; sunset, 8:21 P.M.

species have been found (Russell, 1928), and even between sexes of the same brood. Thus Langford (1938) finds that male *Diaptomus* moves down at the same time that the female is moving up. It is apparent that each plankton form must be investigated separately. While future investigation may show that all vertical migration is due to a single cause, it is equally probable from the data on hand that many different factors are responsible.

It is hoped to continue these experiments with other species of plankton. Isolated observation of the effect of various factors on behavior has no necessary relation to conditions in the field. If vertical migration is first reproduced in the laboratory, one can be reasonably sure that field conditions are being represented. Only then should various factors be altered in order to find the controlling ones.

SUMMARY

1. The natural diurnal vertical migration of the copepod *Acartia tonsa* can be reproduced in the laboratory. Animals kept in tall glass cylinders exposed to window light move up at night and down during the day.

2. Incandescent or fluorescent lights can be substituted for daylight without affecting this behavior, provided that the illumination is oblique to the axis of the cylinders. Migration does not depend upon the area of the source, spectral energy distribution, or total intensity of the light.

3. When illuminated obliquely to the axis of the cylinder, *A. tonsa* sinks downward. This behavior, which is followed by an upward movement in darkness, is believed to account for the normal migration of this form.

4. When the illumination is parallel to the axis of either a vertical or a horizontal tube, *A. tonsa* swims toward the light. Such highly directional illumination is not likely to be encountered in nature, and this behavior is believed to be a laboratory artifact.

5. Observation of individuals shows that the migration consists of a general trend of scattered movements. No difference was found between the sexes.

6. No evidence was found for a diurnal rhythm other than that caused by the normal alternation of day and night. Migration ceases under constant conditions; it can be reversed by illuminating the animals at night and keeping them in the dark by day; and the normal 24-hour behavior can be compressed into four hours.

ACKNOWLEDGMENTS

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ULTRAVIOLET LIGHT AND THE DEVELOPMENT OF FUCUS EGGS AS AFFECTED BY AUXIN AND pH [1]

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INTRODUCTION

It has been shown earlier (Whitaker, 1941) that unilateral irradiation with suitable doses of a number of wave-lengths of ultraviolet light (2345, 2482, 2537, 2654, 2804, 3130, 3660 Å) causes *Fucus* eggs to develop rhizoids on their non-irradiated halves. In the case of the wave-length 2804 Å, it was further shown that the responsiveness of the eggs increases gradually after fertilization, reaching a maximum in about 7 hours, and that the response of a population of eggs is proportional, over a wide range, to the logarithm of the dosage. Sufficiently strong doses inhibit rhizoid formation altogether. In an earlier paper in which these results were reported (Whitaker, 1941), it was suggested that inactivation or destruction of growth substance (auxin) in the eggs might be the principal means of action of the shorter wave-lengths of ultraviolet. This would suppose that moderate doses determine polarity by differentially destroying auxin on one side of the egg, and that strong doses might prevent rhizoid formation by destroying auxin throughout the egg. If this were so, it might be expected that adding auxin (β-indole acetic acid) to the medium after irradiation would tend to counteract the effects of ultraviolet.[2] The present experiments make this test. Experiments are carried out in sea water acidified to pH 6.0 (which approaches the limit of acid tolerance of the *Fucus* egg) as well as in normal sea water at pH 8.0 because the penetration of β-indole acetic acid into the eggs is favored by reduced dissociation in acid medium. Auxin is also more effective physiologically under acid conditions, i.e. in the molecular form (Bonner, 1934).

The effect of pH of the medium on the response of the eggs to ultraviolet was also determined to provide further information about the reactions in the protoplasm caused by the ultraviolet.

[1] This work has been supported in part by funds granted by the Rockefeller Foundation.

[2] This might be expected even if a uniform application of auxin did not abolish a gradient of concentration in an egg irradiated with a moderate dose from one side, if enough auxin is supplied so that the concentration at the irradiated surface reaches or passes an optimum.

The shorter wave-lengths of ultraviolet are heavily absorbed by the protoplasm of the *Fucus* egg (Whitaker, 1941) so that the immediate effect of the ultraviolet must be greatest in the cortical region on the irradiated side of the egg. This raises a question that is broader than the rôle of auxin. Do the rhizoids form on the non-irradiated halves of the eggs because the ultraviolet so damages the cortex upon which it falls that rhizoid formation becomes impossible? If so, the rhizoids would have to form, if at all, on the non-irradiated halves of the eggs, as observed. One series of experiments is designed to answer this question.

Material and Method

Fucus furcatus was collected at Moss Beach and at Pescadero Point, California, from December 1940 to July 1941, inclusive. The gametes were obtained by methods already described, and fertilization was caused to take place at a determined time, $\pm$ 7 minutes. Filtered sea water (Sp. gr. 1.026–1.027) at pH 7.9–8.1 was used as the medium throughout, except where otherwise specifically noted. When sea water was acidified to pH 6.0, approximately one-tenth part of Sorensen's buffer was added and the mixture was aerated with a scintered glass nozzle until pH equilibrium became established. No consequential change in specific gravity resulted. The eggs were reared in a constant temperature room at $15 \pm 1/4°$ C., and were shielded from white light until the final results were observed.

The ultraviolet source was a gas tube mercury resonance lamp made by Westinghouse, and sold under the trade name Sterilamp. The lamp has a 10-inch tube made of Corex glass, which stops almost all of the λ 1800 Å band. The lamp produces practically no heat and more than 90 per cent of the radiant energy is of the wave-length 2537 Å. There are small amounts of λ 3130 and 3660 Å, and some bluish visible light. It has been shown earlier (Whitaker, 1941) that pure λ 2537 Å directs the position of rhizoid formation in *Fucus*, and also that λ 3130 and 3660 are relatively ineffectual compared with the shorter wave-lengths of ultraviolet. For present purposes, therefore, the effects of the radiation from the Sterilamp can be considered quite dominated by the effects of λ 2537 Å.

Four clear fused quartz culture vessels of a considerable degree of optical perfection were made by the Hanovia Chemical and Manufacturing Company. These vessels measured $50 \times 25 \times 15$ mm., and eggs were irradiated and reared in single or double rows on slabs of plate glass resting on the bottoms inside the quartz vessels. When there were two parallel rows of eggs, the second row was placed on a glass shelf

above and behind the first row so that no eggs were eclipsed. In all cases the eggs within the rows were at least 5 egg diameters apart, and the ultraviolet light passed through $0.9 \pm .1$ cm. of medium before reaching the eggs. The culture vessels were maintained on a leveled glass platform so that the eggs did not roll, and a microscope was arranged so that the eggs could be observed at any time with red light without touching the platform or vessels.

The unit of dosage adopted in this work represents the application to the eggs of 1 erg per square millimeter, without correction for scatter and absorption in passing through the vessel wall and 9 mm. of sea water. The scatter and absorption are minor, and one unit very nearly represents the application of 1 erg per square millimeter to the surfaces of the eggs. The intensity of the ultraviolet shorter than λ 3200 Å emitted by the Sterilamp was measured by means of a Hanovia Ultraviolet meter. The intensity of the lamp source under working conditions did not vary more than about 6 per cent. Its intensity was measured from time to time during an exposure, and the variation was compensated for by adjusting the duration of exposure to give the desired total dose.

The distance of the Sterilamp, and the length of lamp tube used, were varied to obtain great range of effective intensity. When the dosage was 1–100 units, the lamp was 4 feet from the eggs; 4 cm. of lamp tube was used and energy was supplied to the eggs at the rate of about 1 unit every 16 seconds. When the dosage was 20,000–50,000 units, the lamp was 6 inches from the eggs, and energy was supplied to the eggs at the rate of about 525 units per minute. Individual shutters were used to interrupt the ultraviolet to each vessel, and when the vessels were relatively near the lamp, parallel side baffles ran from either end of each vessel to the lamp tube so that the eggs did not receive ultraviolet from too great an angular span of directions. The beam of ultraviolet approached the vessels with a downward slant of about 3° to insure against eclipsing by the forward margin of the slab upon which the eggs rested.

When strong dosages (20,000–50,000 units) of ultraviolet were applied, development was greatly delayed. Therefore, after such strong irradiations the eggs were transferred on the glass slabs to glass vessels for rearing, in order to free the quartz vessels for irradiating eggs for new experiments. There may have been some tendency for the eggs to roll during transfer, and partly for this reason the direction of rhizoid formation was not counted in the experiments with strong dosages. The retardation and inhibition of development was measured in these cases.

Results

The Relation between Dosage and Response

Experiments were designed to test the relation between dosage of λ 2537 Å and the response of populations of eggs, in the lower dosage range in which the position of rhizoid formation is determined but development is not impeded. The eggs had been fertilized 8 hours when the irradiation began.

The sensitivity of different samples of eggs varies somewhat, especially as the season progresses. It was therefore necessary to cover nearly the whole span of dosages in each experiment, so that balanced averages might be obtained when all experiments were brought together. Four culture vessels in each experiment were exposed to different dosages, and the average results of 11 experiments are shown graphically in Fig. 1. The points at 1, 2, 5, and 10 units are each based on 550–670 eggs, and the point at 50 units is based on 340 eggs. It is clear that the response of populations is proportional, over a wide range, to the logarithm of the dosage.

This curve (Fig. 1) gives a basis for selecting the dosage for tests of the effect of auxin and pH on the sensitivity of the eggs to ultraviolet. Since 50 per cent of the rhizoids in a population form away from any given side of the culture vessel under conditions of randomness, it is the excess above 50 per cent forming on the halves of the eggs away from the source of radiation that measures the effect of the radiation. Seventy-five per cent represents half of the maximal response to ultraviolet, and while the dosage required to cause this response will vary somewhat, interpolation on Fig. 1 indicates that 2.8 units is a good dosage to use.

Rhizoid Formation on the Surface Irradiated with Ultraviolet

The question was raised at the end of the Introduction whether the irradiated surface of the egg is so damaged by destructive wave-lengths of ultraviolet that rhizoid formation is impossible on this surface. If so, in determining the developmental axis the ultraviolet might merely be acting through general breakdown of a non-specific nature. To find out whether the irradiated surface is capable of rhizoid formation, eggs were first irradiated from one side with 50 and 100 units of λ 2537 Å, since these dosages are more than adequate to cause rhizoids to form on the non-irradiated sides of the eggs (see Fig. 1). The midpoint of the period of irradiation was at 8 hours after fertilization, which is still many hours before rhizoids form (Whitaker and Lowrance, 1936). Immediately after the irradiation with ultraviolet, an ordinary 75-watt

bulb at 1 meter distance was turned on so that the eggs in half of the vessels were irradiated with white light from the opposite side. The white light was left on continuously until results were observed at 26 hours after fertilization. The response of *Fucus* eggs to unilateral irradiation with white light is to form the rhizoids on the sides of the eggs away from the light (Whitaker and Lowrance, 1936). The directional demands of the ultraviolet and of the white light were therefore

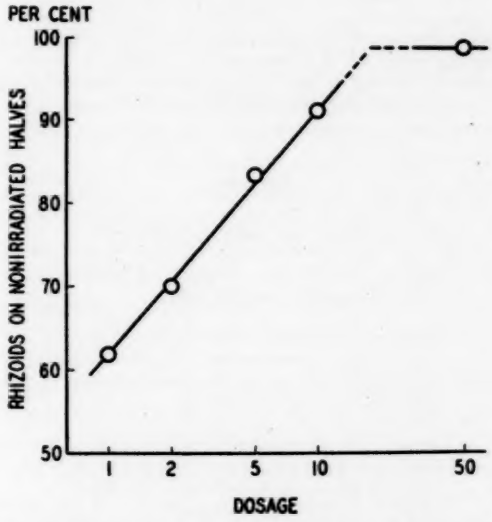

FIG. 1. The vertical axis indicates the percentage of eggs in populations that formed rhizoids on the halves away from the source of radiation after unilateral irradiation with λ 2537 Å. The horizontal axis shows the dosage on a logarithmic scale. One unit of dosage represents approximately 1 erg per square millimeter (see text).

opposed in these experiments, but the white light was left on much longer to dominate.

The results are shown in Table I. It is clear that the white light reverses the direction of rhizoid formation so that rhizoids form on the surfaces of the eggs that were irradiated with ultraviolet. This appears to indicate that the unilateral ultraviolet does not determine the position of rhizoid formation merely by causing extreme destruction and incapacitation of the irradiated surface of the egg. Some more specific effect is to be sought.

The Effect of Auxin on the Response to Low Dosages of Ultraviolet

A series of experiments was designed to test the effect of β-indole acetic acid (auxin) on the sensitivity of eggs to low dosages of λ 2537 Å that determine the polarity of some of the eggs in a population. Eggs were irradiated from one side with 2.8 units at 8 hours after fertilization. In each of 7 experiments two control vessels contained normal sea water throughout, while in two other vessels the medium was changed im-

TABLE I

Results of experiments in which eggs were irradiated from one side with 50 and 100 ergs per square millimeter of ultraviolet light (λ2537 Å). In the second part of each experiment, the irradiation with ultraviolet was followed by a more prolonged irradiation with white light from the opposite sides of the eggs.

Exp.	Total No. Eggs	Treatment	Rhizoids on Halves of Eggs Exposed to Ultraviolet
			per cent
1	238	50 ergs per sq. mm. unilateral ultraviolet	6
	220	50 ergs per sq. mm. unilateral ultraviolet followed by opposed white light	100
2	205	50 ergs per sq. mm. unilateral ultraviolet	2
	192	50 ergs per sq. mm. unilateral ultraviolet followed by opposed white light	100
3	466	100 ergs per sq. mm. unilateral ultraviolet	4
	352	100 ergs per sq. mm. unilateral ultraviolet followed by opposed white light	100
4	209	100 ergs per sq. mm. unilateral ultraviolet	0.5
	176	100 ergs per sq. mm. unilateral ultraviolet followed by opposed white light	100

mediately after irradiation to sea water containing 150 mg. per liter of β-indole acetic acid at pH 8.0. A small amount of NaOH was added to maintain pH at 8.0. This concentration of β-indole acetic acid was the greatest that could be used at pH 8.0 without considerably delaying the development of the eggs. In most but not all of the experiments the eggs in the solution containing β-indole acetic acid gave a somewhat greater response. The overall averages for the 7 experiments, involving 1230 control eggs in sea water and 1200 eggs in the β-indole acetic acid-sea water solution, show that 64 per cent formed rhizoids on the halves

away from the ultraviolet in normal sea water, while 69 per cent did so in the sea water containing β-indole acetic acid. The difference is significant. It can be concluded, however, that the effect of adding β-indole acetic acid, even in amounts beginning to be toxic, is slight, and it does not tend to decrease the effect of the ultraviolet.

Another series of experiments was carried out in which the same concentration of β-indole acetic acid was added some time before irradiation. Its presence greatly decreased the response of the eggs, but absorption measurements showed that this could be explained by the very great extinction of the ultraviolet in passing through 9 mm. of the solution before reaching the eggs.

Since β-indole acetic acid may not enter the eggs readily in its highly dissociated state at pH 8.0 (although it was physiologically active in retarding development), its effect has also been tested at pH 6.0, again using the strongest concentration that did not considerably delay development. The permissible concentration at pH 6.0 was much less: 5 mg. per liter. In both experimental and control vessels the eggs were placed in acidified sea water at 2½ hours after fertilization. They were irradiated with 2.8 units of λ 2537 Å at 8 hours after fertilization, and immediately after the irradiation the β-indole acetic acid was added to half of the vessels. The averaged results of 4 experiments, involving 742 control eggs and 670 experimental eggs to which β-indole acetic acid was added, show that 70.6 per cent of the control eggs formed rhizoids on the halves away from the radiation, and 69.1 per cent of the experimental eggs did so. The results of the individual experiments agree with this average result, and it may be concluded that β-indole acetic acid does not increase the sensitivity of the eggs at all at pH 6.0.

Other experiments were carried out in which the β-indole acetic acid was added some hours before irradiation. These experiments gave similar results, although the presence of even 5 mg. per liter of β-indole acetic acid at the time of irradiation slightly reduced the dosage of ultraviolet received by the experimental eggs, by absorption.

The Effect of Auxin on the Inhibiting Action of Strong Dosages of Ultraviolet

The preceding series of experiments tested the effect of β-indole acetic acid on the sensitivity of the eggs to relatively small unilateral doses of λ 2537 Å, as indicated by the positions at which the rhizoids formed. The object of this series of experiments is to find out whether the effects of heavy doses of λ 2537 Å, which are so strong as greatly to delay and inhibit rhizoid formation and cell division, are mitigated or relieved by

the addition of β-indole acetic acid. If the delay and inhibition of rhizoid formation is due primarily to the destruction of auxin in the egg cells, addition of β-indole acetic acid after irradiation should relieve the symptoms. Four experiments were carried out in which the eggs received a dosage of 20,000 units. The irradiation began 7.7 hours after fertilization and lasted approximately 38 minutes. Immediately after the irradiation, 150 mg. per liter β-indole acetic acid was added to the medium of half the vessels. The pH of all media was 8.1.

Normal non-irradiated eggs have all formed rhizoids at 24 hours after fertilization (Whitaker, 1936). The eggs irradiated with 20,000 units were retarded, however, so that less than half had formed rhizoids at 48 hours when counts were made in the present instance. The averaged results of the four experiments, involving 790 control eggs and 780 experimental eggs to which β-indole acetic acid was added, show that 35.2 per cent of the control eggs had formed rhizoids at 48 hours after fertilization, and 36 per cent of the experimental eggs had done so. The difference is entirely without significance and the conclusion is that β-indole acetic acid does not revive heavily irradiated eggs, or relieve their retardation or inhibition. This conclusion is further supported by the results of a few experiments in which 40,000 and 50,000 units of λ 2537 Å were applied. These heavy dosages greatly retarded and inhibited rhizoid formation, but the addition of β-indole acetic acid (150 mg. per liter at pH 8.0; 5 mg. per liter at pH 6.0) had no effect.

The Effect of pH on the Response to Ultraviolet

The effect of hydrogen ion concentration at the cell surface upon the response to ultraviolet is of interest because it provides further information about the reactions brought about in the protoplasm by the ultraviolet. Therefore experiments were designed to compare the sensitivity of the eggs to λ 2537 Å when the pH of the medium was 8.0 and 6.0. In each of 7 experiments two culture vessels contained eggs in medium at pH 8.0 and two contained eggs in medium at pH 6.0. Eggs were placed in medium at pH 6.0 2½ hours after fertilization, and all eggs were irradiated with 2.8 units at 8 hours after fertilization. In every one of the 7 experiments the eggs in medium at pH 6.0 responded more strongly. The overall average, based on 918 eggs at pH 8.0 and 1033 eggs at pH 6.0, shows that 65 per cent formed rhizoids on the halves away from the ultraviolet at pH 8.0 while 75 per cent did so at pH 6.0. This significant difference shows that the eggs are quite appreciably more sensitive to λ 2537 Å when they are in acidified medium. The eggs used in this and certain other series of experiments were less sensitive than those used to obtain the results shown in Fig. 1, but this is not regarded

as important since changes of this order are commonly found in different collections, especially at different times.

Another group of experiments was carried out to test the effect of pH of the medium on the sensitivity of the eggs to the retarding and inhibiting effects of strong dosages. In three experiments eggs were irradiated with 30,000 units of ultraviolet. The mid-point of the period of irradiation was at 8 hours after fertilization. In each experiment eggs in two vessels were placed in normal sea water at pH 8.0, while eggs in two other vessels were placed in sea water acidified to pH 6.0 at 2½ hours after fertilization and remained in this acidified medium thereafter. In all, 1049 eggs were irradiated and reared at pH 8.0, and 1358 eggs were irradiated and reared at pH 6.0. Non-irradiated control eggs have all formed rhizoids at 24 hours after fertilization (Whitaker, 1936), but the development of eggs irradiated with 30,000 units of λ 2537 Å is strongly retarded and inhibited. At 48 hours after fertilization, 6 per cent of the irradiated eggs at pH 8.0 had formed rhizoids, while 25 per cent of the eggs at pH 6.0 had formed rhizoids. Each experiment separately shows essentially the same relation. One of the experiments was carried further, until eggs that had not formed rhizoids began to cytolyze. In this experiment, at 130 hours after fertilization, 32 per cent of the eggs at pH 8.0 had formed rhizoids while 61 per cent of those at pH 6.0 had done so. In another experiment approximately 400 eggs were irradiated with 50,000 units at pH 8.0, and a like number at pH 6.0. At 96 hours after fertilization none of the eggs at pH 8.0 had formed rhizoids, while 7 per cent of the eggs at pH 6.0 had formed rhizoids.

These results with strong dosages of λ 2537 Å show that the eggs are less sensitive to the damaging effects of strong ultraviolet when in medium at pH 6.0 than they are in medium at pH 8.0. Acidification of the medium thus has an opposite effect on sensitivity to strong damaging doses, compared with its effect on the sensitivity to polarity determination by moderate unilateral irradiation. This opposite effect of acidification suggests that different factors are involved in the two phenomena. Absorption measurements showed that normal sea water and acidified sea water transmit λ 2537 Å equally well, and it should also be noted that non-irradiated control eggs form rhizoids, and early rhizoids grow, at essentially the same rate in medium at pH 8.0 and in medium at pH 6.0.

Discussion

The results presented in Fig. 1 show that the position of the rhizoid formation is extremely sensitive to ultraviolet light of the wave-length

2537 Å, and that the response of populations of eggs is proportional, over a wide range, to the logarithm of the dosage. This relation of response to dosage is similar to that already shown for λ 2804 Å (Whitaker, 1941), and similar to the dosage-response relation in the polarized plasmolysis of *Fucus* eggs after unilateral irradiation with λ 2804 Å (Reed and Whitaker, in press).

Auxin has been found to be present in *Fucus* eggs by du Buy and Olson (1937), and van Overbeek (1940) has shown that auxin occurring naturally in brown algae is very probably β-indole acetic acid. It was pointed out in the Introduction that destructive wave-lengths of ultraviolet might act on the *Fucus* egg primarily by inactivating or destroying auxin, and that, if so, addition of β-indole acetic acid might be expected to counteract or reduce the effect of the ultraviolet. The present experiments show, however, that β-indole acetic acid has very little effect on the sensitivity of the eggs to small doses of ultraviolet at pH 8.0, and none at all at pH 6.0. The slight effect found at pH 8.0 is not in the direction of reducing the effect of the ultraviolet. It may be due to a slight acidifying action of the β-indole acetic acid after entrance into the cell since acidity causes an increased sensitivity. β-indole acetic acid does not relieve or counteract the retarding and inhibiting effects on rhizoid formation and cell division of strong doses of ultraviolet at pH 8.0 or at pH 6.0. It thus appears that the ultraviolet does not act primarily by inactivating or destroying auxin, either in determining the developmental polarity or in inhibiting rhizoid formation.

Acidification of the medium to pH 6.0 appreciably increases the sensitivity of the eggs to the polarity-determining effects of moderate dosages of unilateral λ 2537 Å. On the other hand, acidification of the medium considerably decreases the sensitivity of the eggs to the retarding and inhibiting effects of strong dosages. This suggests that the two phenomena depend on different actions of the ultraviolet.

Summary

1. When *Fucus* eggs are irradiated unilaterally with suitable moderate doses of λ 2537 Å, the rhizoids form on the non-irradiated halves of the eggs.

2. The response of populations of eggs is proportional, over a wide range, to the logarithm of the dosage.

3. When moderate doses of unilateral ultraviolet are followed by prolonged exposures to white light from the opposite sides of the eggs, the rhizoids form on the surfaces that were irradiated with ultraviolet. Therefore, the ultraviolet does not determine the position of rhizoid

formation merely by causing extensive, general damage and incapacitation of the surface upon which it falls.

4. β-indole acetic acid (auxin) has very little effect on the sensitivity of the eggs to moderate doses of ultraviolet at pH 8.0, and none at pH 6.0.

5. β-indole acetic acid does not revive eggs in which rhizoid formation and cell division have been retarded or inhibited by heavy doses of λ 2537 Å. Therefore the ultraviolet does not appear to act on the rhizoid formation primarily by affecting or destroying auxin.

6. The sensitivity of the eggs to the polarity-determining effects of moderate unilateral dosages of λ 2537 Å is considerably increased in sea water acidified from pH 8.0 to 6.0.

7. The sensitivity of the eggs to the retarding and inhibiting effects of strong dosages, on the other hand, is considerably decreased in medium acidified to pH 6.0. The two phenomena (directive effect of small doses and inhibitory effect of large doses) thus appear to depend on different actions of the ultraviolet.

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THE GAS CONTENT OF THE SWIMBLADDER OF THE ROCK BASS, AMBLOPLITES RUPESTRIS, IN RELATION TO HYDROSTATIC PRESSURE

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Introduction

A number of years ago Haldane suggested that the gases in the swimbladder of a physoclistomous fish are of blood origin; i.e., they are secreted into the swimbladder. Evidence has been secured in favor of such an hypothesis by Hall (1924), Powers (1932), Von Ledebur (1937), and Black (1940). However, Krogh (1941) states, "This suggestion is very plausible, but even if proved would not go far to explain the gas secretion in this organ." The work of Powers, Jacobs, and Meesters and Nagel suggests the fundamental rôle of carbon dioxide in the physiology of the swimbladder. Perhaps it is necessary to know the limits of hydrostatic adjustment, the effect of barometric pressure changes on the general physiology of the fish, and the relation between the partial pressures of the gases in the swimbladder and the hydrostatic pressure before the physiology of the rete mirabile and the gas gland can be thoroughly understood. Many facts concerning the physiology of the swimbladder must be known before the rôle of this organ can be definitely established in the adjustment of fish to adverse conditions at certain levels, in the adjustment to barometric pressure changes, and the failure of adjustment in "sudden" mortality of fish during times of lake turnover.

The work presented below was performed with the hope of establishing a basis for further work toward the possible solution of these problems.

Apparatus and Method

An apparatus for the accurate control of hydrostatic pressure was designed, which consisted of a steel tank with a capacity of 32 gallons and capable of withstanding a pressure of 180 pounds. The tank was fitted with two 1½-inch, two 1-inch, and two half-inch tappings, a "sight glass" of vellemoid glass 2½ inches in diameter, and one handhole. The interior of the tank was lighted with a 40-watt bulb inclosed

in an explosion-proof globe purchased from the Crause-Hinds Company of Syracuse, New York. Pressure was maintained by a piston pump, the model " Marvel " constructed by the Deming Pump Company of Salem, Ohio. Under the experimental conditions the capacity of this pump was approximately two gallons per minute.

Pressure below one atmosphere was obtained by connecting the outlet of the tank to the pump, and the inlet to the lake. A by-pass was placed between the tank and the pump. Valves were placed between the tank and lake, tank and pump, and in the by-pass leading to the lake. The tank was filled completely with water, closed, and the pump started

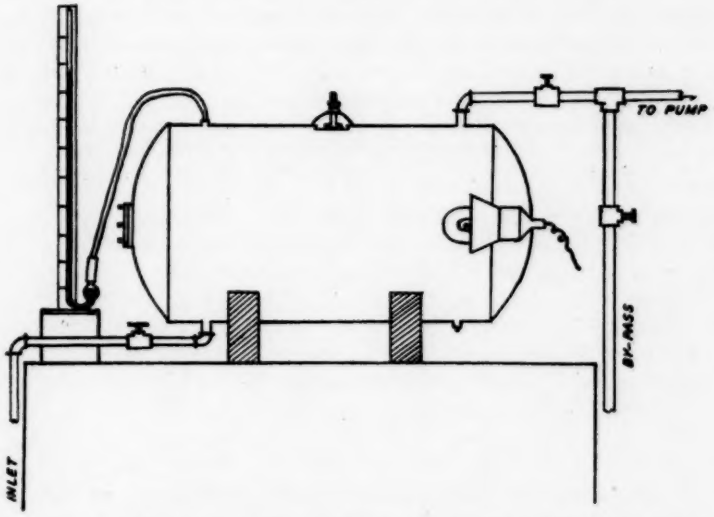

FIG. 1. Diagram of experimental apparatus, showing the arrangement used to produce pressure below one atmosphere.

with the by-pass open. When the pump had reached its capacity the by-pass was closed, and all the water was sucked from the lake through the tank. With this arrangement a pressure of 540 mm. Hg, equal to an altitude of 8,000 ft., was obtained. Figure 1 shows the arrangement of connections necessary to obtain this pressure.

The apparatus was thoroughly checked for air leaks by analyzing samples of water from the tank on the Van Slyke Blood Gas Analysis Apparatus and comparing with the analysis of samples taken from the open lake from which the water was pumped. In no case was there evidence that air was being forced into the water by the pump. The

gaseous content of the lake water remained practically constant throughout the experimental period (See Table I).

Hydrostatic pressure was measured by a gauge which was calibrated before the experiments were carried out and checked again at the end of the work. Low pressures were measured by means of a mercury manometer.

Gas was removed from the swimbladders of the experimental fish by an apparatus which consisted of two tonometers, a leveling bulb, and a hypodermic needle. The second tonometer was filled with mercury, as was the needle and its connection, and a partial vacuum was created in the first tonometer. The hypodermic needle was then inserted into the lumen of the swimbladder, the stopcocks opened, and gas was sucked into the second tonometer. All gas samples were removed while the

TABLE I

Analyses of the oxygen and nitrogen in the lake water compared with the amounts of these gases in the water of the tank at the time of the experiment.

No. Exp.	Average Pressure in Tank	O_2 in Tank	O_2 in Lake	N_2 in Tank	N_2 in Lake
	mm. Hg	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*
6	761	.57	.52	1.13	1.11
12	2218	.77	.73	1.26	1.23
14	2229	.59	.71	1.11	1.18
19	1420	.67	.72	1.12	1.14
20	1147	.60	.60	1.33	1.33
24	1153	.72	.77	1.09	1.12
25	996	.91	.81	1.08	1.08
27	540	.79	.72	1.26	1.30
28	540	.79	.77	1.35	1.32

fish were submerged. The gas was analyzed by the use of the Henderson-Bailey Modification of the Haldane Apparatus.

The rockbass, *Ambloplites rupestris,* was selected as the experimental animal because it has a closed type of swimbladder, survives handling well, and recovers quickly from pressure effects. The blood of this fish is perhaps better buffered than such species as sheepshead and perch (Powers et al, 1939). The rockbass survives relatively high tensions of carbon dioxide.

The experimental procedure was as follows. Three rockbass, which had been removed from a shallow live-box where they had been confined for two days, were placed in the tank and the pressure was gradually increased to the point desired, where it was maintained until the fish had reached a state of hydrostatic equilibrium. This condition is defined as being that state in which the fish make little effort to maintain their

position; i.e., they swim on an even keel. When it was evident that this condition had been established (by observation through the "sight glass"), the fish were removed from the tank and gas samples were taken, usually within two minutes after the fall of pressure.

Discussion of Experimental Data

Determinations of the swimbladder gas contents were made on fish which had been subjected to the following total pressures (hydrostatic plus atmospheric): 540 mm. Hg, 751 mm. Hg, 760 mm. Hg, 996 mm. Hg, 1148 mm. Hg, 1417 mm. Hg, and 2223 mm. Hg. The fish were removed from the tank when they had established a state of hydrostatic equilibrium. Several experiments were carried out at each pressure level, and as there were usually slight differences in pressure, the above pressures are averages. The data of the experiments are tabulated in Table II. The results obtained from the analyses of swimbladder gas taken from fish subjected to 750 and 751 mm. Hg are designated "controls." Those obtained at 760 mm. Hg were the results of analyses of swimbladder gas taken from fish which were removed from a live-box where they had been kept for at least two days. The results at 750 mm. Hg were obtained from fish which had been placed in the tank for six hours with no pressure registering, but with a constant flow of water passing through the tank. The average percentage of carbon dioxide in the swimbladders of fish taken from the live-box was 2.02 per cent, and 1.54 per cent in the case of the fish placed in the tank for six hours. The oxygen percentage in the "control" experiment at 750 mm. Hg was 14.98 per cent, while the percentage of oxygen in the swimbladder of fish taken from the live-box was 15.93 per cent, on the average. The nitrogen in the "control" was 83.63 per cent, and in the fish taken from the live-box the percentage of nitrogen was 81.92 per cent.

At the suggestion of Dr. Alfred C. Redfield, a constant volume of the swimbladder was assumed for fish in a state of hydrostatic equilibrium at any hydrostatic pressure. For example, fish subjected to 750 mm. Hg pressure, the "control," were assumed to have a certain volume of the swimbladder, and fish which were in a state of hydrostatic equilibrium at 996 mm. Hg would have the same volume which they had at 750 mm. Hg, since the density of the fish is equal to the density of the water, and the mass of the fish is unchanged when the fish have reached a state of hydrostatic equilibrium. This assumption gives a basis for calculating the volume of each gas, the gain or loss of each gas in the swimbladder in making the equilibration from the level of the "control," and the rate of gas deposition at each pressure level. In Table III the milliliters of each gas in 100 ml. of swimbladder gas at a given hydrostatic pressure

is corrected to normal conditions of temperature and pressure, that of the "control."

The results at the various pressure levels will be discussed with reference to the volume of each gas at normal temperature and pressure, beginning at the reduced pressure of 540 mm. Hg. When fish had

TABLE II

Results of the analyses of swimbladder gas obtained from the rockbass, Ambloplites rupestris, which had attained a state of hydrostatic equilibrium at the total pressures given in column No. 2.

No. Exp.	Total Pressure	Time	Temp. of Water	CO_2	O_2	N_2
	mm. Hg	*hours*	*° C.*	*per cent*	*per cent*	*per cent*
26	540	24	26.4	0.85	3.94	95.21
27	540	24	26.4	0.73	6.37	92.93
28	540	22.5	25.5	0.75	4.22	95.02
28	540	22.5	25.5	0.72	4.78	94.49
28	540	22.5	25.5	0.72	4.19	95.08
av.	540	23	26	0.77	4.50	94.54
1	750	6.0	11.6	1.23	14.79	83.98
1	750	6.0	11.6	1.54	13.50	84.96
1	750	6.0	11.6	1.79	16.20	82.21
2	751	6.0	13.9	.97	10.01	89.02
2	751	6.0	13.9	1.91	17.98	80.11
2	751	6.0	13.9	1.67	16.30	83.03
3	751	6.0	15.5	1.81	18.94	79.25
3	751	6.0	15.5	1.42	13.10	86.48
av.	750	6.0	13.6	1.54	14.98	83.63
32	759	1.b.*	21.6	1.15	18.17	80.68
32	759	1.b.	21.6	1.87	20.87	77.26
6	761	1.b.	22.2	3.37	13.68	82.44
6	761	1.b.	22.2	1.67	11.02	82.31
av.	760		22.0	2.02	15.93	81.92
25	996	10.5	26.6	6.73	37.11	56.12
25	996	10.5	26.6	3.02	35.08	61.89
25	996	10.5	26.6	4.66	25.86	69.46
av.	996	10.5	26.6	4.73	32.68	61.26

* 1.b., live box.

reached hydrostatic equilibrium at 540 mm. Hg, it was found that all gases had been partially removed from the swimbladder. At this pressure the amount of carbon dioxide in 100 ml. of swimbladder gas was only 0.7 ml., and the amount of oxygen on the same basis was only 4.5 ml., while the nitrogen in 100 ml. of swimbladder gas was 94.5 ml. When corrected to normal conditions of temperature and pressure, the

above volumes become 0.5 ml. CO_2, 3.1 ml. O_2, and 65 ml. N_2. The amounts of each gas in 100 ml. of swimbladder gas removed from fish in the "control" at 750 mm. Hg and 13.6° C. are 1.5 ml. CO_2, 15.0 ml. oxygen, and 83.6 ml. nitrogen. Thus it can be seen that one milliliter of carbon dioxide per 100 ml. of swimbladder gas has been lost along with 11.9 ml. of oxygen and 17.6 ml. of nitrogen. (Table III gives the milliliters of each gas gained or lost per 100 ml. of swimbladder gas in

TABLE II—*Continued*

No. Exp.	Total Pressure	Time	Temp. of Water	CO_2	O_2	N_2
	mm. Hg	*hours*	*° C.*	*per cent*	*per cent*	*per cent*
20	1147	24.1	25.5	16.45	35.67	47.88
21	1145	23.7	26.1	3.50	45.28	51.20
21	1145	23.7	26.1	8.29	44.50	47.20
21	1145	23.7	26.1	9.36	34.38	55.81
24	1153	25.1	25.5	15.24	35.22	49.45
24	1153	25.1	25.5	11.88	36.92	51.13
25	1153	25.1	25.5	4.46	39.60	55.94
av	1147	24.4	25.5	9.88	38.86	51.24
19	1420	38.5	26.1	6.69	51.60	41.71
19	1420	38.5	26.1	13.24	46.89	39.79
19	1420	38.5	26.1	11.80	32.17	55.83
5	1414	47.5	22.2	15.27	42.24	42.23
5	1414	47.5	22.2	11.27	40.89	46.84
5	1414	47.5	22.2	2.35	56.85	40.80
av.	1417	43.0	24	10.17	45.10	44.53
12	2218	56.5	21.6	8.43	55.57	36.00
12	2218	56.5	21.6	7.40	61.30	31.31
12	2218	56.5	21.6	8.53	60.26	31.14
13	2223	56.5	21.6	7.97	62.17	29.92
13	2223	56.5	21.6	10.99	42.07	46.94
14	2229	66.5	25	9.02	52.05	35.35
14	2229	66.5	25	9.75	45.90	43.35
14	2229	66.5	25	8.39	61.18	40.48
av.	2223	61.0	22.6	8.96	54.73	36.31

equilibrating to the various levels of hydrostatic pressure.) In order to reach equilibrium at 540 mm. Hg, approximately 27 per cent of the gas in the swimbladder at equilibrium at 750 mm. Hg would have to be removed. Since only 5 per cent of the gas at 750 mm. Hg is carbon dioxide and oxygen, equilibrium at 540 mm. Hg cannot take place unless nitrogen is removed. Unless nitrogen is removed, the rockbass could not come to equilibrium at a pressure of less than 635 mm. Hg. Brown (1939)

offers experiments on the guppy as evidence of nitrogen deposition at reduced pressures. The results on the rockbass do not show nitrogen deposition in the adjustment to any pressure, as will be pointed out below. However, Brown found that the guppy did not make adjustment to pressure reductions of more than 125 mm. Hg. If the swimbladder, as stated above, did not contain more than 16 per cent carbon dioxide plus

TABLE III

The milliliters of each gas gained or lost per 100 ml. of swimbladder gas were calculated from the data secured at each pressure level as given in Table II, assuming a constant volume of the swimbladder at hydrostatic equilibrium.

Average Total Pressure	CO_2	O_2	N_2	
mm. Hg	*ml*	*ml*	*ml*	
540	0.7	4.5	94.5	per 100 ml.
	0.5	3.1	65.0	corrected NTP
	−1.0	−11.9	−17.6	gain or loss
750	1.5	15.0	83.6	"Control" NTP = 750 mm. Hg, 13.6° C.
760	2.0	15.9	81.9	per 100 ml.
	1.9	15.6	86.4	corrected NTP
	+0.4	+0.6	−3.2	gain or loss
996	4.7	32.7	61.3	per 100 ml.
	6.0	41.5	78.8	corrected NTP
	+4.5	+26.5	−4.8	gain or loss
1147	9.9	38.9	51.2	per 100 ml.
	14.5	57.3	75.3	corrected NTP
	+13.0	+42.3	−8.3	gain or loss
1417	10.2	45.1	44.5	per 100 ml.
	18.3	81.9	80.6	corrected NTP
	+16.8	+66.9	−3.0	gain or loss
2223	8.9	54.7	36.3	per 100 ml.
	25.6	156.5	103.8	corrected NTP
	+24.1	+141.5	+20.2	gain or loss

oxygen, adjustment could not be made to pressures below 635 mm. Hg without the removal of nitrogen.

In the adjustment to 996 mm. Hg it was found that 4.5 ml. carbon dioxide and 26.5 ml. oxygen were deposited in the swimbladder while 4.8 ml. nitrogen were removed for every 100 ml. of swimbladder gas.

At hydrostatic equilibrium at the total pressure of 1147 mm. Hg there were deposited in the swimbladder 13 ml. carbon dioxide and 42.3 ml. oxygen; and 8.3 ml. nitrogen were removed per 100 ml. of swimbladder gas.

The adjustment to the hydrostatic pressure of 1417 mm. Hg was the result of the deposition of 16.8 ml. carbon dioxide and 66.9 ml. oxygen per 100 ml. of swimbladder gas, while 3 ml. of nitrogen were removed.

Likewise there was a deposition of 24.1 ml. carbon dioxide and 141.5 ml. oxygen per 100 ml. of swimbladder gas in the adjustment to 2223 mm. Hg pressure. In this case nitrogen appeared to have increased. The slight deviation of the nitrogen in several experiments from the level of the "control" is believed to be due to an error in the use of average figures, originally caused by the failure of some fish in the experiment to have reached complete equilibrium.

Figure 2 is a graphic representation of the data compiled in Table III in which the milliliters of the three gases per 100 ml. of swimbladder gas are plotted against the hydrostatic pressure in mm. Hg. It can be seen that the nitrogen fluctuates only slightly. It will be noted that the volume of carbon dioxide plotted against the hydrostatic pressure forms a curve which tends to level off as the pressure increases. The oxygen forms a nearly straight line, which indicates that the deposition of this gas is largely responsible for the adjustment to hydrostatic pressure. At the total pressure of 2223 mm. Hg, over half of the pressure exerted against the swimbladder wall is due to oxygen. Carbon dioxide partial pressure (200 mm. Hg) is equal to only one-tenth of the total pressure. Meesters and Nagel (1934–35) state that 86 per cent of the "Ersatzegase" is composed of carbon dioxide. Their results were obtained for only the first hour of gas deposition. If the gas entering the swimbladder is composed of 86 per cent carbon dioxide for the duration of gas deposition, carbon dioxide partial pressure would be much higher than 200 mm. Hg. In as much as the pressure of oxygen at 2223 mm. Hg is half the total pressure, it is impossible that the secretion of carbon dioxide continued to form 86 per cent of the total secretion and it is quite likely that the ratio of carbon dioxide to oxygen secreted changes greatly during equilibration. The ratio of these two gases varies considerably at equilibrium. In Table IV the milliliters of carbon dioxide and oxygen are the corrected volumes of these gases in 100 ml. of swimbladder gas observed at equilibrium at the various pressure levels.

The average ratio is 6.5; i.e., there are 6.5 times more oxygen than

carbon dioxide in the swimbladder at hydrostatic equilibrium. Meesters and Nagel found 6.1 times more carbon dioxide than oxygen in the gas which entered the swimbladder during the first hour of gas deposition.

Two series of experiments were performed which cast some light on the rate of gas deposition, the rôle of carbon dioxide after the first two hours of gas deposition, and upon the relation of the amount of carbon

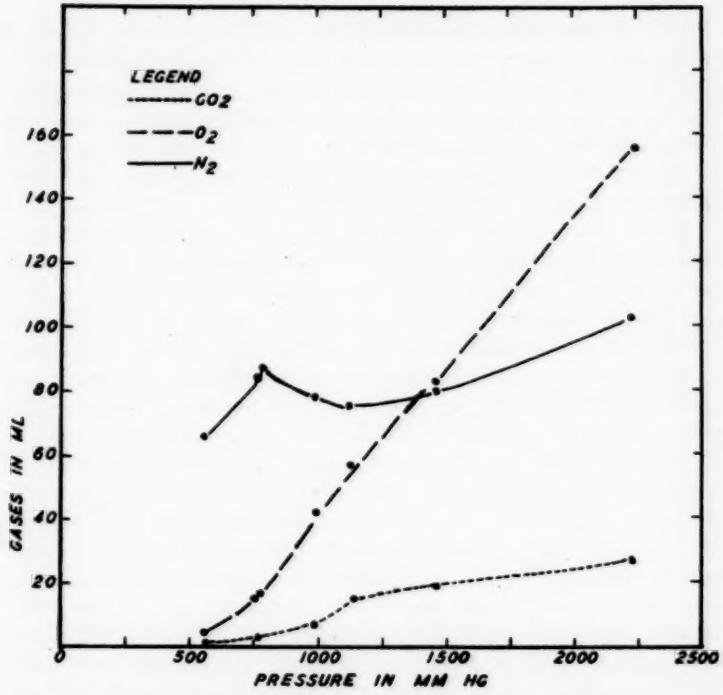

FIG. 2. A graphic representation of the data of Table III wherein the volume of gas is calculated on the assumed volume of 100 ml. for the swimbladder at hydrostatic equilibrium.

dioxide deposited to the strength of the stimulus—the hydrostatic pressure. In one series of experiments the pressure, 996 mm. Hg, remained constant, and the fish were placed under this pressure for various lengths of time. The results of analyses of swimbladder gas taken from fish which had been subjected to 996 mm. Hg for two hours, 10.5 hours, and

TABLE IV

Hydrostatic Pressure	CO_2	O_2	Ratio O_2/CO_2
mm. Hg	*ml.*	*ml.*	
540	0.5	3.1	6.2
750	1.5	15.0	10.0
760	1.9	15.6	8.2
996	6.0	41.5	6.9
1147	14.5	57.3	3.9
1417	18.3	81.9	4.4
2223	25.6	156.5	6.1

24.6 hours are given in Table V. Figure 3 is a graphic representation of these data wherein the partial pressures of the various gases are plotted against "time." It can be seen that the partial pressure of carbon dioxide is rapidly increased, from the point of the "control" during the first two hours. The "control" is the average amount of carbon dioxide in the swimbladder of fish at the surface of the lake. The rate of increase of carbon dioxide falls off during the next eight hours. When the fish are allowed to remain at this pressure (996 mm. Hg) for 24.6 hours, which is 12.5 hours after they have reached a state of hydrostatic equilibrium, carbon dioxide partial pressure has decreased by

TABLE V

These data were obtained from analyses of swimbladder gas taken from fish which had been subjected to 996 mm. Hg hydrostatic pressure for the durations listed in column No. 2.

No. Exp.	Time	Total Pressure	CO_2 Content	CO_2 Pressure	O_2 Content	O_2 Pressure	N_2 Content	N_2 Pressure	Temp. of Water
	hours	*mm. Hg*	*per cent*	*mm. Hg*	*per cent*	*mm. Hg*	*per cent*	*mm. Hg*	°C.
29	2.0	993	7.65	74.2	25.70	248.3	66.66	644.0	26.8
29	2.0	993	3.42	33.0	17.30	167.1	79.26	765.7	26.8
29	2.0	993	1.01	9.7	26.60	256.9	72.39	699.3	26.8
av.			4.03	39.0	23.20	204.5	72.81	703.0	
25	10.5	996	6.73	64.6	37.11	353.6	56.12	559.3	25.8
25	10.5	996	3.02	29.0	35.08	337.1	61.89	594.7	25.8
25	10.5	996	4.66	44.7	25.86	248.5	69.46	677.5	25.8
av.			4.73	46.1	32.68	325.1	61.26	610.5	
23	24.6	999	2.13	20.8	31.27	305.9	66.60	651.5	22.7
23	24.6	999	3.81	37.2	35.22	344.5	60.96	596.4	22.7
23	24.6	999	2.06	20.1	45.45	444.5	52.46	513.2	22.7
av.			2.67	26.0	37.33	365.0	60.00	587.0	

two-fifths of its value at 10.5 hours. Concomitantly with the rise in carbon dioxide, there is an 8 per cent increase in oxygen during the first two hours of stimulation. During the next eight hours the oxygen increased 12 per cent, while only 2 per cent increase was shown for this gas during the last 14 hours. It has been suggested by Dr. Laurence Irving [1] that the delay in the maximum of oxygen in contrast to carbon dioxide in the swimbladder may be due to the relatively large volume of

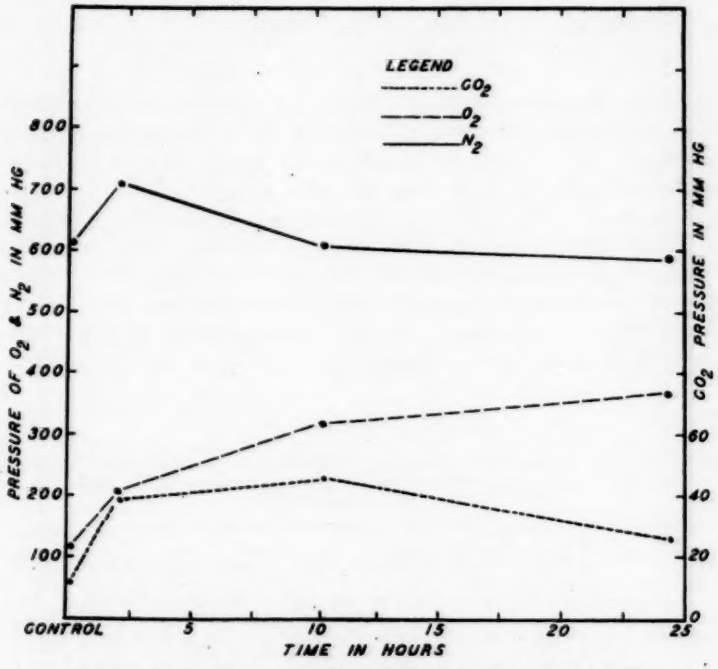

FIG. 3. A graphic representation of the data given in Table V.

oxygen which must be moved. The partial pressure of the nitrogen falls to the level of the atmospheric nitrogen by the end of the 24-hour period. Thus it can be seen that nitrogen is not deposited. Oxygen and carbon dioxide are rapidly deposited during the first two hours, and unless carbon dioxide is being removed from the swimbladder as it is being deposited, oxygen is entering the swimbladder as rapidly as

[1] Dr. Laurence Irving by personal communication.

carbon dioxide, if not more so, during the first two hours. It hardly seems likely that carbon dioxide is being removed from the swimbladder during the early part of adjustment, for, in the light of Meesters' and Nagel's and Jacob's work, carbon dioxide deposition may account for small adjustments to hydrostatic pressure. Carbon dioxide could not diffuse out of the bladder rapidly during this length of time, since it has been proved by Meesters and Nagel (1934–35) that the "oval" is tightly closed during the early part of gas deposition. Fish subjected to a total pressure of 996 mm. Hg reach hydrostatic equilibrium in 10 hours. After this time carbon dioxide partial pressure decreased toward the level of the carbon dioxide partial pressure of the control. It appears that carbon dioxide is being removed from the swimbladder after the fish have attained a state in which they are capable of moving about without too much difficulty.

In another series of experiments fish were subjected to 1417 mm. Hg for various periods of time, from 2 hours to 73.5 hours. The results of the analyses of swimbladder gas are given in Table VI, and graphically illustrated in Fig. 4, in which the partial pressures of the gases are plotted against "time" in hours. The carbon dioxide increased sharply during the first two hours from 1.54 per cent of the control to 7.98 per cent at the end of the two-hour period. During the next five hours the carbon dioxide increased to 13.93 per cent, which is the highest partial pressure recorded for this gas at the total pressure of 1417 mm. Hg. From this time, 7.5 hours, to the longest period of adjustment, carbon dioxide percentage decreased to the lowest point, 4.22 per cent, in 73 hours. This percentage is equal to a partial pressure of 59.1 mm. Hg, which is still higher than the partial pressure of this gas in the control. This indicates that, even in 73 hours, fish which have been subjected to 1417 mm. Hg of pressure have not reached a state of gaseous equilibrium within the swimbladder. It is evident that after 38 hours carbon dioxide is being removed faster than it is entering the swimbladder. The highest average partial pressure for carbon dioxide in the swimbladder of fish subjected to 1417 mm. Hg is 192 mm. Hg, and the partial pressure of CO_2 in the swimbladder of fish subjected to 2223 mm. Hg of total pressure is 197 mm. Hg. Since the carbon dioxide curve levels off at 200 mm. Hg, it might be possible that this is the limit of the amount of carbon dioxide this particular species can deposit. In other words, the level of the carbon dioxide partial pressure may be a limiting factor to the hydrostatic adjustment. The highest average partial pressure for carbon dioxide in the swimbladders of fish subjected to 996 mm. Hg is 46.1 mm. Hg. Thus it can be seen that there

TABLE VI

These data were obtained by the analyses of swimbladder gas taken from fish which were subjected to 1417 mm. Hg hydrostatic pressure for the duration listed in column No. 2.

No. Exp.	Time	Total Pressure	CO_2 Content	CO_2 Pressure	O_2 Content	O_2 Pressure	N_2 Content	N_2 Pressure	Temp. of Water
	hours	*mm. Hg*	*per cent*	*mm. Hg*	*per cent*	*mm. Hg*	*per cent*	*mm. Hg*	*°C.*
22	2.0	1415	8.82	122.6	36.92	513.2	54.26	754.2	26.1
22	2.0	1415	8.10	112.6	35.49	493.3	56.41	784.1	26.1
22	2.0	1415	6.04	83.9	19.20	266.9	74.16	1031.0	26.1
av.			7.98	106.3	30.53	424.4	61.61	856.4	
18	7.5	1415	13.72	190.3	30.05	416.8	56.10	778.1	28
18	7.5	1415	8.72	120.9	38.02	527.3	53.26	738.7	28
18	7.5	1415	19.17	265.9	26.87	372.7	53.93	748.0	28
av.			13.93	192.3	31.96	438.9	54.39	754.9	
17	13.7	1415	13.75	191.3	25.07	348.7	60.98	848.2	25.6
17	13.7	1415	11.06	153.8	23.67	329.3	65.27	908.3	25.6
17	13.7	1415	13.12	182.5	32.43	451.1	54.21	754.1	25.6
av.			12.67	175.8	27.09	376.3	61.14	836.9	
4	27.0	1416	13.70	196.3	30.86	430.8	55.44	773.9	22.2
4	27.0	1416	13.10	182.9	30.38	424.1	56.52	789.0	22.2
4	27.0	1416	11.10	154.5	38.44	536.6	50.86	710.0	22.2
av.			12.60	176.2	33.23	463.8	54.26	757.6	
19	38.5	1420	6.69	93.3	51.60	719.8	41.71	581.9	26.1
19	38.5	1420	13.24	184.7	46.89	655.8	39.79	565.1	26.1
19	38.5	1420	11.80	164.6	32.17	448.8	55.83	778.8	26.1
av.			10.38	147.5	43.55	606.1	45.77	641.9	
5	47.5	1414	15.27	212.8	42.24	588.8	42.23	588.7	22.2
5	47.5	1414	12.27	171.0	40.89	570.0	46.84	652.9	22.2
5	47.5	1414	2.35	32.7	56.85	792.5	40.80	576.6	22.2
av.			9.96	138.8	46.66	650.4	43.30	606.7	
10	66.0	1426	4.83	67.9	57.71	811.7	37.46	527.0	21.6
10	66.0	1226	10.06	141.4	48.90	688.0	41.04	577.3	21.6
av.			7.45	104.7	53.20	749.8	39.35	552.1	
30	73.3	1425	2.68	37.6	54.51	764.6	42.81	600.5	23.9
30	73.3	1425	8.42	118.1	42.72	598.3	48.70	683.3	23.9
30	73.3	1425	1.56	21.8	51.79	726.6	46.67	654.8	23.9
av.			4.22	59.1	49.73	696.5	46.06	646.2	

is a relationship between the partial pressure of carbon dioxide and the stimulus, the hydrostatic pressure. In the experiments carried out at 1417 mm. Hg the oxygen increased sharply during the first two hours with the initial increase in carbon dioxide, but, as in the experiments at

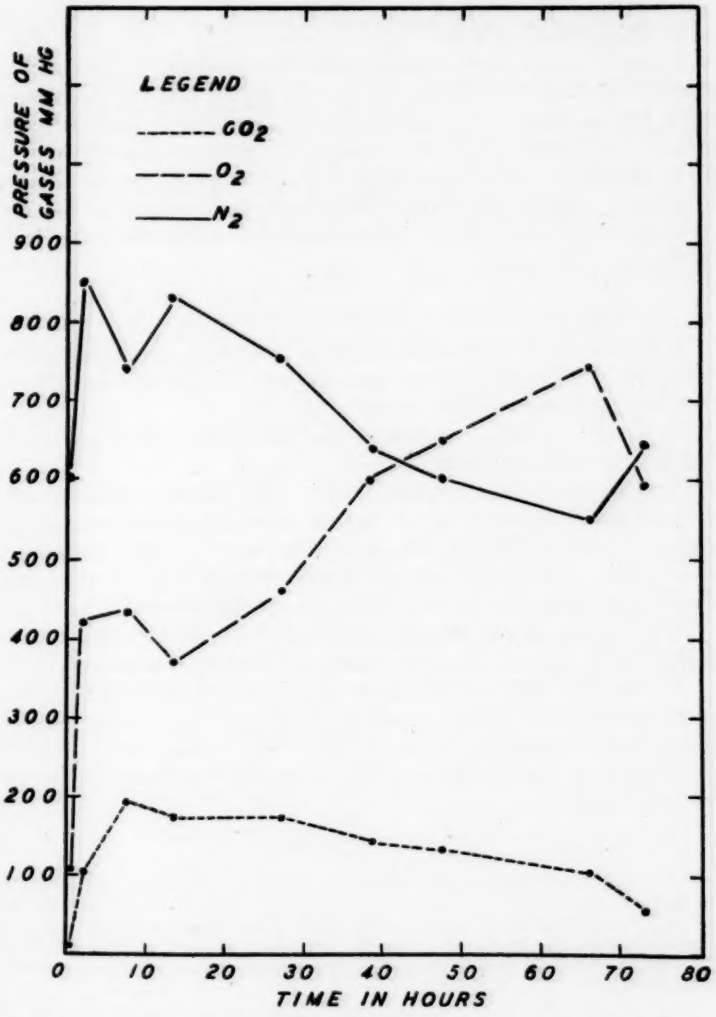

FIG. 4. A graphic representation of Table VI. The point 0 represents status of swimbladder gas of the control.

996 mm. Hg, the oxygen continued to increase after the carbon dioxide is decreasing. The nitrogen partial pressure decreases toward the level of the atmospheric nitrogen.

From these two series of experiments it is evident that both carbon dioxide and oxygen are increased sharply during the first two hours. After the fish have made a temporary adjustment to hydrostatic pressure, carbon dioxide is removed from the swimbladder faster than it is being deposited. The final adjustment results in a high partial pressure of oxygen, a carbon dioxide partial pressure near the level of the control, and a nitrogen pressure approximately equal to the atmospheric nitrogen partial pressure.

Summary

1. Rockbass were subjected to various hydrostatic pressures ranging from 540 to 2223 mm. Hg and were observed to reach a state of equilibrium at each experimental pressure.

2. Oxygen deposition accounted for half of the adjustment at the higher hydrostatic pressures.

3. After hydrostatic equilibrium had been reached carbon dioxide diffused out of the swimbladder, and the carbon dioxide percentage decreased toward the level of this gas usually found in fish at the surface of the lake. This loss in volume by the outward diffusion of carbon dioxide was compensated for by the continued deposition of oxygen.

4. Nitrogen was not deposited, and appeared to be only passively important in the adjustment to the changes in hydrostatic pressure.

5. The final adjustment to hydrostatic pressure resulted in a high partial pressure of oxygen, a carbon dioxide partial pressure near the level usually found for this gas in fish at the surface, and a nitrogen partial pressure approximately equal to the partial pressure of this gas in the atmosphere.

ACKNOWLEDGMENTS

The author is indebted to Dr. Raymond C. Osburn, under whose direction this work was carried out, and to Dr. Gordon C. Ring and Dr. Edwin B. Powers for advice and encouragement; also to Ohio State University and Franz Theodore Stone Laboratory for the use of equipment. He wishes to thank Dr. Alfred C. Redfield and Dr. Laurence Irving for reading the manuscript and for their subsequent suggestions.

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BUDS INDUCED IN CLYMENELLA TORQUATA BY IMPLANTS OF NERVE CORD AND NEIGHBORING TISSUES DERIVED FROM THE MID-BODY REGION OF WORMS OF THE SAME SPECIES

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In an endeavor to learn more concerning the factors involved in the determination of the type of structure which will develop when a part of the body is lost, a variety of transplantation experiments have been carried out on the polychaete. *Clymenella torquata.* It has been shown that when pieces of nerve cord with neighboring tissues from non-regenerating donors are inserted in the body wall of intact hosts, buds are frequently induced. An implant derived from the anterior end (segments 1–7) of a donor may give rise to a bud of either head or tail type (Sayles, 1940*a*). Whether a head or a tail forms depends to a large extent upon the segment of the host into which the implant is inserted. In the region between segments 1 and 9, head buds have been induced by anterior implants in about one case in six, but no tail buds have ever appeared. When inserted posterior to segment 12, this type of implant does not produce head buds but often results in the formation of an incomplete tail bud with the ventral part of the anal segment missing. The region of segments 10–12 is a transitional zone in which several types of well-differentiated buds may appear, including: (1) heads; (2) tails with the ventral parts of the anal segments missing as at posterior levels; (3) buds each with the ventral half of the anal segment missing and with a cone of new material projecting posteriorly into the open region; (4) buds each with a terminal region consisting of the dorsal half of an anal segment on the open, ventral side of which is a proboscis or similar part.

Implants taken from the posterior region of donors, on the other hand, will not produce head buds, regardless of the level of the host's body at which they are inserted. At posterior levels they produce complete tails. In the transitional zone they give rise to two definite types: (1) complete tails and (2) buds terminating in anal segments of which

only the ventral parts are present. At anterior levels these implants also frequently produce buds terminating in ventral parts of anal segments. In some cases the latter bear extra, conical parts dorsally.

Both anterior and posterior implants exhibit a definite polarity. Definitive head or tail buds are ordinarily not induced by anterior implants when they are inserted with their posterior ends exposed at the surface of the host's body. Posterior pieces, however, must be oriented with their posterior ends out if they are to result in differentiated buds.

The present paper, reporting on results obtained with implants derived from the mid-body region, is intended to complete the description for transplantation experiments in which both donors and hosts are non-regenerating worms. There are reported 277 cases in which implants taken from this mid-body region, segments 8–11, were inserted in the three different regions of the body of *Clymenella*. As in previous experiments, the implants were inserted in the dorso-lateral parts of the segments. This work was carried on at the Marine Biological Laboratory, Woods Hole, during the summers of 1935–1940, inclusive. In the following account most of the buds are described as they were at the time of preservation or discard or, in some cases, a brief time before they were lost.

TABLE I

Results of implantation of mid-body pieces with anterior end exposed.

Level of insertion	Anteriorly			In mid-body			Posteriorly			Totals
Source of implant, segments	8–9	9–10	10–11	8–9	9–10	10–11	8–9	9–10	10–11	
Head buds	2	2		4	3	1				12
Double Tail			1							1
Tails, open ventrally				2	1		2	3	1	9
Tails, complete							5	1		6
Indeterminate buds	7	4	3	10	6	7			4	41
No new material	7	6	7	2	3	2	1	4	6	38
Died within 10 days	2	3		4	3	1	1	4	3	21
Implant lost by host	1	2	1	1				1	1	7
Totals	19	17	12	23	16	11	9	13	15	135

RESULTS

The results of 135 cases in which the anterior ends of the implants were exposed are summarized in Table I. The double tail bud consisted of a basal, rounded region bearing two small, partial, anal segments open

ventro-laterally, with the open sides turned slightly toward one another. Each of the buds designated "tail, open ventrally" terminated in a partial anal segment with the ventral half missing. In most of the buds of this type formed at posterior levels the anal-segment regions were large with only a very small mid-ventral region missing in each case. Buds described as "tail, complete" were so called because the anal segments were complete. The indeterminate buds included all of those not possessing at least a clear-cut portion of a head or tail segment. Some of these were elongate with more or less evidence of segmentation but with rounded terminal regions. Others were somewhat conical masses of new material, with occasionally one or more notopodial or neuropodial elements present.

TABLE II

Results of implantation of mid-body pieces with posterior end exposed.

Level of insertion	Anteriorly			In mid-body			Posteriorly		Totals
Source of implant, segments	8–9	9–10	10–11	8–9	9–10	10–11	9–10	10–11	
Head buds		1	2			2			5
Head-tail buds			1			1			2
Tail-cone bud			1						1
Tail, open dorsally			1		1	5			7
Tail, complete						4	3	10	17
Indeterminate buds	5	3	7	9	5	16	6	3	54
No new material	8	7	3	2	3	7	3	1	34
Died within 10 days	2	3	2	3	2	5	2	1	20
Implant lost by host	1			1					2
Totals	16	14	17	15	11	40	14	15	142

In the remaining 142 operations the posterior ends of the implants were at the surface of the host's body. These are summarized in Table II. Each of the head-tail buds terminated in a weakly differentiated peristomial region and had a partial anal segment, open dorsally, attached in the ventral region of the main bud a short distance from the peristomium. The tail-cone bud was unsegmented and terminated in a large, partial anal segment, the dorsal side of which was missing. On the dorsolateral side of this anal segment, near its base, there was a small, conical mass of material.

From these data it is clear that implants derived from the level of segments 8–9 possess a distinct polarity. Although 15 definitive head or tail buds resulted from 51 operations in which the anterior ends of these implants were out, none of the 31 cases in which the posterior ends

of the implants were at the surface produced a head or tail bud. It is also interesting that implants from 8–9 produced no tails when inserted at anterior levels, no heads at posterior levels and both types in the transitional region. Both of the mid-body tails were formed at the tenth segment. In all of these respects these implants from segments 8–9 follow rather closely the behavior of implants from anterior sources.

Implants from segments 9–10 of donors, when oriented with their anterior ends out, produced results which resembled those of pieces made up of segments 8–9. The only marked difference was in the distinctly smaller number of tail buds produced by segments 9–10 at posterior levels of hosts. But these implants when inserted with their posterior ends out produced several well-differentiated buds. One was a fairly well formed head which developed at an anterior level, segment 7. One, formed at segment 10, was a small tail bud with only the ventral part of the anal-segment portion present. The other three, produced at segments 13 and 14, were small tail buds with complete anal collars. In distinct contrast with these results are those obtained with anterior pieces inserted with their posterior ends out, an arrangement in which definitive head or tail buds are very rarely produced.

Implants from segments 10–11 showed further transition toward the behavior typical of posterior pieces. Definitive head or tail buds appeared in 3 of 38 cases (about 8 per cent) in which the anterior ends of the implants were exposed. These were of three different types: a double tail, a fairly well-differentiated head, and a bud with only the dorsal part of the anal segment present. On the other hand, 27 out of 72 cases (about 37 per cent) in which the posterior ends of such implants were on the surface gave rise to head or tail buds. At anterior and mid-body levels these belonged to several different types. At posterior levels, however, only tails with complete anal segments were formed. Also the number of differentiated buds produced was much greater in proportion at posterior levels (67 per cent) than in the anterior or transitional regions.

Discussion

In general these results with implants from mid-body sources are in accord with what one might expect from a study of buds formed from implants from the anterior and posterior regions of donors. In each case where a tail bud with partial anal segment arose from an implant inserted with its anterior end out, the dorsal part of the anal segment was the portion present regardless of the source of the implant. Evidence has been presented (Sayles, 1940*b*) that the ventral part of the

terminal segment is usually determined by the implant and the dorsal part usually by the host. It is clear, then, that although a partial tail bud may be induced at the anterior end of an implant, the nature of this structure is probably determined by the host. In the case of a partial tail bud produced by an implant with its posterior end out, it was always the dorsal part of the anal segment which was missing. These implants from the mid-body region showed further that the type of bud produced is determined through the interaction of implant and host.

When inserted anterior to the tenth segment, anterior implants produced heads but never tails (Sayles, 1940*a*). Posterior implants inserted in this anterior region produced no heads but frequently tail buds, all of whose anal segments lacked dorsal portions (Sayles, 1940*b*). Mid-body implants made up of the nerve cord of segments 8–9 or 9–10 gave results similar to those following the implanting of anterior pieces here. Pieces of nerve cord made up of segments 10–11, however, showed intermediate behavior. They produced both tails and heads, with the latter being formed even when the posterior ends of implants were exposed. This appearance of heads would not have been predicted from the study of implants from anterior and posterior sources.

In the mid-body region anterior pieces produced both head and tail buds with the latter frequently bearing extra parts which in some cases showed definite head features. Posterior pieces, however, were virtually limited to the formation of tail buds without extra parts. Pieces of nerve cord made up of segments 8–9 or 9–10 gave rise to both head and tail buds, with the latter not bearing extra parts. Again, at this level, implants from segments 10–11 produced both heads and tails.

At posterior levels implants from both anterior and posterior sources produced only tail buds, which in the majority of cases were complete. Similar results were obtained with pieces from the mid-body region.

These results seem to be best explained on the basis of the following theory. There is a strong tail-determining factor in the posterior part of the body. Whenever this is present at full strength, any well-differentiated bud formed will terminate in at least a partial anal segment. This factor is weak or lacking in the anterior part of the body. There is also a head-determining factor, present in the anterior part of the body but either absent in the posterior part of the body or overpowered there by the much stronger tail-determining factor. In the mid-body region (segments 9–11) there is a transition between these conditions found at anterior and posterior levels. This transition may in some way be associated with a general change in the body at the 9–10 intersegmental level. At this point there is a sharp line of demarcation anterior

to which the integument is thicker and richer in glands and the longitudinal muscle bundles are much larger than in the region posterior to this level. No doubt there also exist other, less obvious differences which may be related to the determination of regenerating or induced buds.

That the tail-determining factor at maximum strength is distinctly stronger than the head-determining factor at its maximum is shown by two types of experiments. Implants from the anterior, head-forming region, inserted in the posterior tail-forming part of the host, induce only tail buds, never heads. Implants from the posterior region, when inserted in the anterior part of hosts, also induce only tail buds.

It seems advisable to leave any further discussion of the significance of these results, using implants from the several regions of intact donors, until after presentation of data from experiments already completed using regenerating worms as donors or hosts. Reports on this work with regenerates are now being prepared for publication. An abstract (Sayles, 1941) covering part of the material has already appeared.

Summary

The data presented in the present paper and in the two related papers previously published (Sayles, 1940*a* and *b*) may be summarized as follows.

When oriented with their anterior ends out: (1) implants which include the eighth or more anterior segments will produce definitive head or tail buds with the type dependent to a considerable extent upon which segment of the host is involved; (2) implants including only segment 9 or segments 9–10 will also produce both general types of buds but probably not as frequently as implants from more anterior sources; (3) implants from segment 10 or segments 10–11 occasionally form a head or tail bud; (4) posterior implants which do not include any material from a level anterior to segment 11 very rarely, if ever, produce determinate buds.

When oriented with their posterior ends out: (1) anterior implants involving no cord posterior to the ninth segment produce no head or tail buds; (2) implants including only segment 10 or segments 9–10 rarely give rise to these buds; (3) implants including only segment 11 or segments 10–11 give rise to head and tail buds in many cases; (4) posterior implants including only segment 11 or more posterior segments produce tail buds frequently, but head buds rarely, if ever.

These data indicate that: (1) There is a tail-determining factor which is strong in the posterior half of the body but weak or absent at the

level of segments 1–8. (2) There is a head-determining factor which is strongest in the anterior region of the body, weaker in the mid-body region, and perhaps finally absent in the posterior part of the worm. (3) The tail-determining factor at maximum strength is distinctly stronger than any head-determining factor in *Clymenella*.

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CROSS- AND SELF-FERTILIZATION IN THE ASCIDIAN STYELA

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In 1904 I made a number of cross- and self-inseminations of *Cynthia partita* (*Styela*) at Woods Hole. Single isolated individuals gave the following percentages of self-fertilization: 33, 10, 100, 95, 95, 75, 10, 30, 30, 10, 1, 75, 90, 85, 33, 0, 4, 4, 0, 12, 4. Many other individuals did not spawn. The results ranged from perfect self-fertility to absolute self-sterility. Several experiments were also made with eggs and sperm artifically removed. There was less selfing and not very much cross-fertilization. Owing to the relatively large amount of body fluids that comes over with the eggs, and the large number of immature eggs, the method is inadequate for the best results. This applies also to other tests in which ether, alcohol and ammonia were added to the mixture of eggs and sperm. In most cases more eggs were selfed than in the controls in sea water. It may be noted that when these substances were later used on *Ciona* eggs, they likewise gave erratic results of doubtful significance; but later (1939) it was found that treatment of the eggs with a dilute solution of HCl for 2 to 5 minutes, after washing in sea water and selfing, gave consistently 100 per cent normal cleavage and often normal embryos. I found that the same HCl solutions did not increase the amount of self-fertilization in *Stylea;* but it is not improbable, I think, that self-fertilization could be brought about by HCl here also if somewhat different treatment were followed.

In 1930 Plough reported for *Styela* (*Cynthia*) that no selfing occurs in some individuals while other individuals show almost 100 per cent self-fertility. The amount of self-fertilization was determined by a "count of the tadpoles—usually not yet hatched—which were present on the following morning." These counts generally exceeded the number of eggs that had early cleaved. "Thus the full number of eggs which develop was secured, and abnormal cleavages eliminated." In other words, the data do not give the actual number of eggs fertilized but only those that gave normal tadpoles. In the experiments he reported, one lot of eggs (A) was completely self-sterile, while another

lot (K) gave 42 per cent self-fertilization. Cross-fertilization of (A) gave 16, 20, 17, 46 per cent (not 56 as stated in the text), while cross-fertilization of (K) gave 40, 69, 42, 42 per cent.

Plough reported that by adding 4 drops of N/10 NH_4OH to samples of the above eggs the percentage of selfing for K rose to 77 per cent, and the cross lot Ka rose from 36 to 79 per cent.

During the following summer, 1931, I repeated Plough's method with NH_4OH on a rather large scale and failed to get any evidence that self-fertilization was increased in *Styela*.

In 1932 Plough repeated and extended his former experiment and found that most frequently the treated eggs gave a slightly reduced percentage of fertilization both in selfed and crossed samples. The difference between the earlier and later results he explains as due to the eggs of the former record having lost their resistance to their own sperm by standing in sea water before fertilization, since there was an interval of 45 minutes that "elapsed between the fertilization of the first set of egg samples (K) and that of the second (K′) in which self sterility was found to have disappeared. In this year's tests the egg samples were prepared at the same time and fertilized almost simultaneously." It should be noted, however, that self-sterility in (K′) had not "disappeared" but only 79 per cent of the eggs selfed which was, however, about the same percentage as those that crossed. That more eggs were selfed than those observed to cleave in the normal time is a fact that I have also found in *Ciona*. Frequently the number of tadpoles that appear in a dish of selfed eggs is greater than the number of cleavages recorded after one hour. It is not improbable that some of the increase is due to loss of resistance to selfing on standing, but there is another possibility that cannot be overlooked. There may be present in the sperm suspensions a relatively smaller number of sperm that differ genetically from the majority, and it may take time for them to meet the eggs and fertilize them. This is consistent with the evidence for *Ciona* that the greater the initial amount of sperm, the larger the number of eggs that are self-fertilized. The same relation was noted by Plough. Now in *Styela* the eggs and sperm are set free at the same time, and on removing some of the eggs to another dish of sea water some sperm will as a rule go over with them. If the sperm suspension carries over some of the exceptional spermatozoa, a certain amount or all of it will be used up for self-fertilization, but if more sperm is added either at the time or later, a larger number should be fertilized. The increased number of self-fertilizations that takes place after a time can be explained on this assumption.

Plough points out further that certain of his experiments "suggest that cross fertility may increase during the first three hours after shedding although much less rapidly than self-fertility." He tested this as follows. All of the eggs were removed from dishes into which they were shed and squirted into a larger amount of fresh sea water. "This treatment brings about very considerable dilution of the suspension of own sperm surrounding the eggs and a truer picture of the effect of standing on cross fertility may be expected." He found that selfing increased during two hours but cross-fertility gradually diminished and later rapidly fell off to zero. These results are also consistent with the suggestion that I have made above. Cross-fertilization decreases on standing because the sperm (not the eggs) rapidly lose their fertilizing power. This would be expected also for the selfed lots, but by diluting the sperm it would take a longer time for the relatively fewer number of sperm that can enter their own eggs to reach them. In Plough's table to illustrate his hypothesis there was an increase of 7 to 24 per cent in the selfed lots, i.e., to the extent of three times the amount of selfing.

In 1932 Plough reported at the Sixth Genetic Congress but no new data are given. In 1933 he reported that during the past three summers he and Miss Alderman have been studying *Styela* and have found that decrease and increase of the hydrogen concentration of the sea water, etc., . . . "only reduce fertility all along the line." By the use of crab digestive fluid, the membrane was removed. "There is always a general effect when this fluid is used to digest off the membranes but in the best cases many eggs develop. Strangely enough, removal or weakening of the membrane has no effect on the relation of self and cross fertilization. The membranes appear to have nothing to do with the block to self fertilization in Styela." No data are given. He reports that, in sections of selfed eggs, many sperm are found inside the membrane and test cells, and in close contact with the egg cortex, although self-fertilization has not taken place. "Thus self-sterility in Styela seems to be a true case of selective fertilization." It should be recalled that the only spermatozoa that normally fertilize the eggs of *Styela*, as shown by Conklin, are at the antipole, hence, even if spermatozoa are present at the surface of the egg, they would not necessarily be expected to enter the egg. It is conceivable, too, that passage through the egg membrane and test cells would render the sperm incapable of entering the egg except those that enter at the antipole. In 1935 Plough found that *Styela plicata* and *S. barnharti* give the same results as *S. partita*. Eggs exposed to their own sperm show that the sperms penetrate the membrane and come into contact with the eggs. "The data suggest that self

TABLE I

Unfertilized Eggs	Tadpoles	Abnormal Tadpoles	Abnormal Embryos	Counted
52	69	0	45	1/2
0	2	22	200	1/4
502	2	0	0	all
323 (mostly eggs)	13	0	3 (one abn.)	all

sterility in *Styela* results from an individually specific blood block to fertilization by the animal's own sperm." No details are given.

During the summer of 1941 I made some further experiments with *Styela* at Woods Hole. The surface of each was scrubbed clean and the animals were kept in a dish with running sea water until 5 or 6 p.m. when they were put each into a dish ("finger-bowls") of sea water (about 200 cc.) and watched from 8 to 10 p.m. in order to record those that laid eggs. As a rule, only a few of the isolated animals set free sperm and eggs. The animals were then put back together into a large dish of running sea water, and the next day, and sometimes on a third day, were again tested for spawning. The dishes in which eggs and sperm had been set free were examined the next morning and the number of unfertilized eggs, of normal swimming tadpoles, of abnormal tadpoles and of abnormal embryos were recorded. It is difficult sometimes to distinguish between the unsegmented (unfertilized) eggs and the abnormal embryos that had only passed through the segmentation stages, but most of the abnormal embryos show a black "eye spot" that clearly indicates they had begun to develop, but had not passed into later stages. As an example of the relative number of those that set free eggs and sperm on one occasion, 14 were tested on July 23–24. Only four spawned. The animals had been brought in July 21. The record of the four that shed eggs and sperm was as follows (see Table I). The number of the eggs, embryos, etc., actually counted is indicated by the fraction in the last column.

TABLE II

Eggs	Tadpoles	Abnormal Tadpoles	Abnormal Embryos	Counted
364	0	0	0	3/4
0	0	0	149	all
0	37	17	510	4/5
0	59	0	37	?

Individuals of the same lot were put out again (July 24–25). Of these 25 did not lay, but one gave 958 eggs, 7 tadpoles and 1 abnormal tadpole. There were also a few abnormal embryos.

Eighteen of the same lot were set out the next day (July 25). The old *Styelas* were left in the dish. Only four spawned. The results are given in Table II.

A fresh lot was brought in July 26 and set out (July 27). The *Styela* remained over night in the dishes. No spawning took place in 25 dishes; in eight dishes spawning took place. The results are given in Table III.

Thirty-three, including the same individuals, were set out July 28, and 16 of them set free eggs and sperm. The old *Styela* had been removed from the dishes. The results are given in Table IV.

TABLE III

Eggs	Tadpoles	Abnormal Tadpoles	Abnormal Embryos	Counted
691	5	0	6	2/3
404	23	64	265	1/3
49	23	64	153	1/4
479	2	0	2	1/2
79	354	23	434	9/10
709	0	0	0	1/8
96	0	0	0	9/10
253	9	16	55	1/4

In addition to the preceding records, there were 19 other individuals that spawned, but since they gave the same kinds of results, it does not seem necessary to record them here.

Taking the last four tables together, there are 25 cases in which some or all of the eggs were selfed. The ratios of unfertilized to those that were fertilized, including tadpoles and abnormal embryos, varied widely. At one extreme (eight cases), all were self-fertilized; at the other (24 cases), some or many eggs were not self-fertilized.

In practically every case in which the number of fertilized eggs is sufficient to give a significant figure, there was a relatively large percentage of the eggs which did not give normal tadpoles. It is not possible to compare these percentages with those reported by Plough, since he states that he counted only the normal (?) tadpoles that had not yet hatched. It is true that it is possible to record the normal tadpoles with long coiled tails just before hatching, but obviously this in most cases

does not give the percentage of fertilization, whether self- or cross-, but only the percentage of normal development.

To what extent leaving the old *Styela* in the dish may affect the development, since it would be passing water in and out of its siphons, was looked into by comparing sets of dishes in which the *Styela* had been left in over night with those in which they had been removed as in the preceding tables.

In the following test (see Table V) the old Cynthias had been left in the dishes.

In a later test (August 23–24) forty dishes were put out (5 p.m.) and the old *Styela* left in each dish until 10 a.m. the next morning. In 19

TABLE IV

Eggs	Tadpoles	Abnormal Tadpoles	Abnormal Embryos	Counted
595	5	1	0	2/3
0	92	224	672	1/4
some eggs	38	40	most abn.	1/6
259	12	0	0	3/4
103	3	0	92	1/2
528	0	0	0	1/5
41	12	26	109	1/5
423	0	0	0	1/8
405	24	100	29	1/10
312	5	0	4	1/4
0	11	1427 and abn. embryos		1/10
0	419	92 " "	"	1/5
325	518	638 " "	"	1/4
0	29	970 " "	"	1/10
0	633	21 " "	"	1/2
rare	302	342 " "	"	1/5

dishes of 40 set out the individuals had spawned. The record is given in Table VI.

A comparison between these records and those of Table III shows practically no difference in the relative numbers of fertilized eggs, of normal tadpoles and abnormal embryos in dishes (containing about 200 cc. sea water) in which the old *Styelas* were left and in those in which the *Styelas* were removed soon after spawning.

In the next lot of freshly brought-in *Styelas* (July 29) only one individual spawned, giving the following data:

Eggs	Tadpoles	Abnormal Tadpoles	Abnormal Embryos
114	243	108	156

TABLE V

Unfertilized Eggs	Tadpoles	Abnormal Tadpoles	Abnormal Embryos	Counted
382	1	0	0	1/2
302	30	4	0	9/10
700	186	18	25	1/4
493	0	0	1	1/2
361	19	0	0	1/2
601	36	0	17	1/3
273	37	0	9	1/2
475	51	1	31	1/3
354	205	0	34	1/4
241	28	0	7	1/2
508	36	20	87	1/15
2	78	20	87	1/2
324	251	4	18	1/5
555	20	0	7	1/8
87	11	31	333	1/10

The same individuals were tested a day later (July 30). Selfing and reciprocal fertilizations were made between pairs of individuals. Thus *A* eggs were selfed with *a* sperm, and also crossed with *b* sperm. Similarly for *B*. The percentage of self-fertilized eggs (tadpoles and ab-

TABLE VI

Unfertilized Eggs	Tadpoles	Abnormal Tadpoles	Abnormal Embryos	Counted
433	63	2	8	1/3
294	50	0	10	1/5
6	333	0	0	1/5
74	19	0	179	1/5
111	146	2	106	1/4
34	60	10	439	1/4
503	91	0	3	1/2
197	73	0	9	2/3
0	47	96	128	1/5
118	120	45	25	1/8
286	85	0	7	1/7
135	40	0	10	1/3
297	0	0	0	1/8
33	179	0	51	1/5
53	66	0	87	1/3
357	120	0	—	1/8
202	89	7	23	1/2
169	147	—	26	1/2
9	87	1	2	1/1

normal tadpoles and abnormal eggs) is given in the fifth column of Table VII and the proportion of the eggs counted is given in the last column (approximately).

The percentages of eggs that were self-fertilized varied from 1 to 100 per cent. All the eggs that were cross-fertilized gave 100 per cent both ways, and gave practically 100 per cent normal tadpoles. In con-

TABLE VII

	Eggs	Tadpoles	Abnormal Tadpoles	Abnormal Embryos	Percentage Fertilized	Counted
A eggs self	813	98	0	0	10.8	1/4
A eggs by b sperm	0	363	0	2		3/4
B eggs self	0	171	0	326	100	1/2
B eggs by a sperm	0	153	0	0		4/5
C eggs self	82	449	0	41	85.7	1/2
C eggs by b sperm	0	448	0	0		1/2
D eggs self	0	429	68	470	100	1/10
D eggs by c sperm	0	628	0	0		1/3
E eggs self	97	24	14	539	85.6	1/5
E eggs by f sperm	0	135	134	0		1/5
F eggs self	5	513	0	23	99.1	1/6
F eggs by e sperm	0	161	0	0		1/2
G eggs self	0	593	0	14	100	1/5
G eggs by f sperm	0	512	0	2		1/3
H eggs self	0	545	0	10	100	1/4
H eggs by i sperm	0	431	2	0		—
I eggs self	261	143	2	0	36	1/2
I eggs by h sperm	0	389	1	1		9/10
K eggs self	156	99	0	0	39	9/10
K eggs by l sperm	0	2	290	0		9/10

trast, three of the selfed lots produced more abnormal embryos than normals, but the other seven produced fewer abnormals than normals.

The *Styelas* which made the following records (see Table VIII) had been collected August 2. Nine spawned. As soon as this took place some of the eggs, and the sperm (*A*) that went along with them, were put into two dishes of fresh sea water. To one of these some of the sperm suspension of another dish (*B*) was added. The eggs and sperm

that were left in the original dish are designated "selfed." The experiment was made to find out the proportion of eggs fertilized (as judged later by the number of tadpoles and embryos that developed), and also to find out whether the number of selfed eggs in fresh sea water gave a

TABLE VIII

	Eggs	Tadpoles	Abnormal Tadpoles	Abnormal Embryos	Percentage Selfed	Counted	Percentage of abnormal
A eggs selfed	244	62	9	16	26	1/4	28.8
A eggs in fresh sea water	20	14	10	16	67	1/2	53.
A eggs by b sperm	1	258	0	81		1/2	
B eggs selfed	19	92	135	572	98	1/15	88
B eggs in fresh sea water	5	50	5	108	97	1/2	69
B eggs by a sperm	0	558	0	33		1/2	
C eggs selfed	47	234	9	411	93	1/2	64
C eggs in fresh sea water	12	43	1	117	93	2/3	83
C eggs by d sperm	0	93	0	210		3/4	
D eggs selfed	287	15	0	103	32	1/3	87
D eggs in fresh sea water	218	23	0	16	15	1/2	41
D eggs by c sperm	2	71	3	149		1/2	
E eggs selfed	868	0	4	4	0.9	1/10	100
E eggs in fresh sea water	528	0	0	0	0.0	1/5	0
E eggs by f sperm	0	29	16	149		1/2	
F eggs selfed	0	156	4	372	100	3/4	70
F eggs in fresh sea water	0	171	6	87	100	7/8	35
F eggs by e sperm	0	333	8	142		1/2	
G eggs selfed	220	10	0	1	4.8	1/2	9
G eggs in fresh sea water	12	1	0	0	7.7	1/1	0
G eggs by h sperm	27	93	1	27		9/10	
H eggs selfed	190	439	37	11	72	1/3	9.8
H eggs in fresh sea water	20	84	4	8	83	9/10	12
H eggs by g sperm	7	172	1	3		1/2	
K eggs selfed	410	37	9	16	13.1	1/3	40
K eggs in fresh sea water	56	1	0	0	1.8	1/1	0
K eggs by l sperm	23	61	2	1		—	

higher percentage of normal tadpoles than those left in the original dish. The data also show whether the cross-fertilized eggs gave rise to more normal tadpoles than do the self-fertilized eggs. Of course, some or many of these eggs may have selfed before the foreign sperm was added.

Cross-fertilizations were made in pairs (see below) for comparison with each other. The percentages of selfing are given in the fifth column; and the percentages of fertilized eggs that developed abnormally are given in the sixth column.

The percentages of eggs that selfed in the original dish, as compared with those that were removed to fresh sea water, are smaller in only three cases. In two cases the percentage was greater but not much greater; in one case nearly the same (98 and 97 per cent), and in another the same (93 and 93 per cent). There was probably much more sperm in the original dish and also many more eggs, but the percentages of the two may be compared. On the whole, the eggs developed as well in the original dish (from which the *Styela* was removed) as in the dish of fresh sea water.

The number of abnormal embryos is quite high in all the dishes. The percentages of abnormals in original and fresh water dishes were: 28.8–53; 88–69; 64–83; 87–41; 100–0; 70–35; 9–0; 9.8–12, 40–0. Rejecting the fifth and ninth pair, since the numbers are too small to be significant, the counts show that there is no consistent relation of normals to abnormals in the two kinds of dishes; sometimes one, sometimes the other is the greater. In other words, the original dishes of eggs and sperm did about as well as those with fresh sea water in them and fewer eggs. The latter contained eggs, some or all of which may have been fertilized before transfer to fresh sea water, but this would not affect the kind of development that took place.

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CROSS- AND SELF-FERTILIZATION IN THE ASCIDIAN MOLGULA MANHATTENSIS

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The rule for the "single" ascidian *Ciona intestinalis* is no self-fertilization and 100 per cent cross-fertilization, but there are rare cases of the reverse and it is these that may give a clue to the genetic problem involved. Another single ascidian, *Cynthia*, has been shown to self-fertilize frequently but no cases of cross-sterility have been reported so far. A third single ascidian, *Molgula manhattensis*, has been shown to give a high percentage of self-fertilization, mainly from my own earlier examination (*Jour. Exper. Zool.*, 1904), but no thorough comparison of the extent of selfing with the extent of crossing has been made. It seemed worth while to undertake such an examination in the hope that some degree, at least, of self-sterility might be found and, if so, fall in line with the situation in the other two species. In my former paper there were cases of 100 per cent self-fertilization and also a few in which no selfing occurred.

During September, 1941, *Molgula* being abundant on the float in the eel pond at Woods Hole, a number of them were tested. There is an ovotestis on one side, in a loop of the intestine. It is smaller than the one on the other side, and its central slate-colored ovarian portion is, as a rule, more nearly surrounded by the white testis than is the ovotestis on the other side, and this makes it more difficult to remove the ripe eggs without contamination from the testis. The larger ovotestis on the opposite side is generally only partly surrounded by the testis, and the eggs can frequently be removed by a small puncture in the ovarian portion without contamination.

Each individual was first washed in tap water, the test was split open, and the interior mass removed and also washed. A small puncture in the ovarian part allows the eggs to exude. They were then collected in a dish of sea water (15 cc.).

In a preliminary test of six individuals (August 28) three lots of eggs gave no cleavage, showing that no sperm had come out with the eggs, or at least not sufficient sperm for selfing. Three sets gave a high percentage of cleavage, which must have been due to undetected sperm that

had come out with the eggs. The three sets of eggs that had not cleaved were cross-fertilized and all cleaved.

In later experiments the eggs were first removed from an individual (*A*) to a Syracuse dish of sea water; one-half of these was transferred to another dish and later cross-fertilized with sperm from the "reciprocal" individual (*B*). More eggs and some sperm were then removed together from the same individual to another dish. The latter were heavily inseminated with their own sperm. At the same time similar dishes were prepared from individual (*B*), some of whose eggs were crossed with sperm from (*A*). If none of the unfertilized eggs in *A* and *B* cleaved, it would mean that no spermatozoa (or too small an amount to be effective) have been introduced into this dish. Hence, if any or all eggs in the cross-fertilized dish cleaved this was due entirely to cross-fertilization. If any or all eggs cleaved in the self-fertilized dish, this must have been due to its own sperm. As an illustration, the results for three such tests were as follows:

(A) The unfertilized eggs of *A* gave no cleavage; those selfed gave 25 per cent cleavage; those crossed by *B* gave 100 per cent.

(B) The unfertilized eggs of *B* gave no cleavage; those selfed gave 100 per cent; those crossed by *A* gave 100 per cent.

(C) The unfertilized eggs of *C* gave no cleavage; those selfed gave no observed cleavage (but later one coiled embryo was present and a few abnormal embryos); those crossed by *D* gave 100 per cent.

(D) The unfertilized eggs of *D* gave no cleavage; those selfed gave 1 per cent; those crossed (to *C*) gave 75 per cent.

(E) The unfertilized eggs of *E* gave 10 per cent cleavage; hence to this extent some of the "cross-inseminated" eggs may have been selfed before foreign sperm was added; the selfed eggs gave 100 per cent; and the crossed eggs (by *E*) gave 100 per cent.

(F) The unfertilized eggs of *F* gave no cleavage; the selfed eggs gave no cleavage; those crossed to *E* gave 100 per cent.

It is evident from these tests that the amount of selfing that occurred varied from 0 to 100 per cent, while the cross-fertilized eggs gave 100 per cent cleavage except in one case (75 per cent).

Two of the preceding combinations (A–D) are recorded in the first two pairs of Table I. This table gives the results from 38 pairs (or 76 individuals). The respective pairs used in reciprocal crossing are bracketed. With three exceptions the cross-fertilized eggs gave 100 per cent cleavage. The three exceptions (75, 50, 80) were in different pairs. There was nothing peculiar in the selfing of these three cases unless it be

TABLE I

Control	Self	Cross	Control	Self	Cross	Control	Self	Cross
0	25	100	5	100	100	50	100	100
0	100	100	0	100	100	0	100	100
0	0	100	0	100	100	0	2	100
0	1	75	2	75	100	0	40	100
0	100	50	90	100	100	0	90	100
0	0	100	0	80	100	100	100	100
0	35	100	100	100	?	0	12	100
0	100	100	0	0	2	30	100	100
0	100	100	0	100	100	50	100	100
0	10	100	2	75	100	0	0	100
0	90	100	90	100	100	0	100	100
0	15	100	0	80	100	80	100	100
50	100	100	0	50	100	4	8	100
50	50	100	100	100	100	0	8	100
20	20	?	15	15	100	0	0	100
4	14	100	70	80	100	0	20	100
0	100	95	0	0	100	1	0	100
0	20	90	0	10	100	20	75	100
0	80	80	0	10	100	20	100	100
0	80	100	0	20	100	100	100	100
15	25	100	5	100	100	100	100	100
70	80	100	0	100	100	0	100	100
0	0	100	2	5	100	100	100	100
0	10	100	0	100	100	4	100	100
0	10	100	20	100	100			
0	0	100	100	100	100			

that the 50 per cent crossed eggs gave 100 per cent selfed. In addition there were two cases in which, by oversight, the crossed eggs were not recorded, but there can be no doubt that they gave 100 per cent cleavage or attention would have been drawn to them.

An examination of this table shows that no cleavage took place in 44 lots of eggs removed from the ovary and not inseminated. There were 32 other lots with percentages ranging from 1 to 100 per cent selfing. This means that the amount of sperm that had been unintentionally re-

moved with the eggs was too small in amount in most cases to self-fertilize any of the eggs. An examination of the table shows that, in cases where no cleavage occurred in the dishes of control eggs, it also did not take place in 9 of the selfed dishes, which must mean that 10 per cent of the individuals were totally incapable of self-fertilization. Of those lots in which none of the control eggs cleaved (44 cases), and some eggs at least cleaved when selfed, the percentage of eggs cleaved ranged from 2 to 100 per cent. This may be interpreted to mean that although plenty of spermatozoa were present, only a percentage of them were actually effective as measured by the cleavage. I interpret this to mean that in addition to the spermatozoa having the same genetic composition as the eggs, there are varying percentages of sperm with a different genetic composition, and these are able to fertilize eggs from the same individual. This interpretation is also consistent with the evidence from those cases where a small percentage of the eggs in the control uninseminated dishes cleaved. As the table shows, there were 20 cases in which the control "unfertilized" eggs that cleaved gave a lower percentage of cleavage than did the "self-fertilized" eggs, and only 10 cases in which they were the same. These results are consistent with the assumption above, viz., that there are present a smaller and variable number of spermatozoa whose genetic make-up differs from the majority of sperm, the latter incapable of fertilizing their own eggs, but the former having the ability to do so. This conclusion is consistent with the more completely worked-out case of *Ciona*.

It should be noted that I did not come across a single case of cross-sterility, but the numbers may have been too small to expect to meet such cases. There is one case recorded where no eggs were selfed and only two eggs cleaved when cross-fertilized, but I am inclined to think that the sperm was not in condition to self or to cross. It is noticeable here that there is a question mark in the table when the sperm of this individual was used for crossing the reciprocal.

Normal and Abnormal Development

In some of the preceding experiments the dishes were examined when the tadpoles were due to hatch (or after that time) in order to find out whether the development was normal or abnormal. The observations are not here reported since it became apparent that unless the embryos are followed closely before and at the time of expected hatching and later, the records of what constitutes abnormals cannot be accurately defined. For instance, the tadpoles are due to emerge ten to twelve hours after fertilization depending on temperature. Besides being a

rather inconvenient hour for taking records, many of the embryos without passing through a free swimming stage undergo metamorphosis in place, and the time may extend over a considerable interval. This makes it difficult to record normal versus abnormal embryos. In general, it may be said that in a large number of cases there were many abnormal embryos as compared with the number of those that cleaved. This applies mainly to embryos that did not pass into the complete tadpole stage. Those that underwent a metamorphosis without passing through the free-swimming tadpole stage were so variable that without fuller records it is not possible to make a clear statement about them. Further observations are needed. There was no evidence that the selfed lots behaved differently from the cross-fertilized lots.

Two Unisexual Molgulas

Two individuals were found that had no testis on either side but well-developed ovaries. These came from a small bunch of some 35 to 50 individuals, and may therefore have been related. They were full-sized individuals and normal in all other respects. Eggs were taken out separately from each side of each individual. One-half of the eggs of each were kept in case undetected sperm was present, but none of them cleaved. The cross-fertilized eggs taken from one side of one individual cleaved irregularly. Later they gave rise to a few tadpoles and some metamorphosing embryos. The eggs from the other side when crossed gave abnormal cleavage (polyspermic?) and later a few metamorphosed individuals. Eggs taken from the other individual and not fertilized did not cleave. Those from one side that were cross-fertilized gave 100 per cent cleavage and later many metamorphosed individuals. Those from the other side that were fertilized gave 90 per cent cleavage and later a few normal tadpoles and many metamorphosed embryos. The eggs of these two individuals when cross-fertilized did not behave differently from those of bisexual Molgulas. If a new generation could be reared from such unisexual forms, it might be possible to find out if the condition is inherited.

Summary

Tests of self- and cross-fertilizations of the single ascidian *Molgula manhattensis,* were made during August and September, 1941, at Woods Hole. It was found that 100 per cent self-fertilization occurred in less than half the cases (see Table I), but in others the percentages ranged from 1 to 90 per cent. One hundred per cent of cross-fertilization oc-

curred in practically all cases. The occurrence of normal and abnormal development was observed, and many cases of abnormal embryos as well as normal tadpoles were found, but, owing to the fact that many embryos metamorphosed directly, or at least without passing through a free-swimming tadpole stage, the numerical results are not reported here. A closer set of observations are needed to give reliable data as to whether self-fertilized eggs give rise to such development more often than do cross-fertilized eggs. Two mature individuals were found that had only ovaries. Their eggs gave 100 per cent cross-fertilization.